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## INTRODUCTION

Intracellular single chain antibodies (intrabodies) represent novel anti-tumor agents capable of achieving a highly specific, targeted anti-neoplastic effect. In this regard, intrabody knockout of a variety of oncoprotein targets has accomplished diverse anti-neoplastic effects, including cell-specific toxicity, chemosensitization and radiosensitization. In the present proposal we sought to evaluate the utility of the intrabody approach to achieve knockout of the cyclin D1 oncoprotein in the context of carcinoma of the breast. Specific steps were endeavored to develop anti-cyclin D1 intrabodies, validate functional knockout of the target oncoprotein and to adapt this approach as a practical intervention for carcinoma of the breast. These steps were largely completed during years 1 and 2 of this award, and have been previously reported.

Of note, however, phenotypic knockout with the anti-cyclin D1 intrabody appeared to induce only particle phenotypic changes. We hypothesized that the intrabody affinity for its target might represent a predicate of intrabody efficacy. To this end, during the year 3 period we explored this hypothesis using the erbB-2 oncoprotein as a "model" target. It was hoped that "augmented affinity" intrabodies would have an increased capacity for achieving oncoprotein targeted functional knockout. This accomplishment would thus, potentially, predicated augmented therapeutic efficacy of the intrabody approach.

## **BODY OF WORK**

The cancer gene therapy of mutation compensation is based upon rectification of the genetic lesions etiologic of neoplastic progression.<sup>1</sup> For the context of dominant oncogenes, methods have been developed to achieve abrogation of the dysregulated cellular pathways.<sup>2-6</sup> Such interventions have been endeavored at the transcriptional level of gene expression employing triplex, antisense or ribozyme approaches.<sup>4-8</sup> In addition, functional knockout at the level of protein expression has been achieved via dominant negative mutation, as well as intrabody methods.<sup>9-16</sup> This later approach is based upon intracellular expression of single chain antibodies (scFv) to sequester oncoproteins during their biosynthesis, thus preventing their further maturation.<sup>16-20</sup>

On this basis, intrabody knockout of oncoproteins has found broad utility for a variety of molecular targets. In this regard, intrabody knockout of diverse classes of oncoproteins has been reported including overexpressed growth factor receptors (erbB-2, EGF-R), cell cycle proteins (cyclin D1, p53), viral oncoproteins (ras, HBV, LMP1, HPV E6/E7) and anti-apoptosis proteins (Bcl-2).<sup>13;14;16-25</sup> In this context, diverse antineoplastic effects mediated by intrabodies have been reported including tumor cell specific cytotoxicity, chemosensitization and radiosensitization.<sup>24-27</sup> These utilities have

predicated the evaluation of intrabodies in the context of a human clinical gene therapy trial whereby an anti-erbB-2 intrabody has been studied in a phase I protocol for erbB-2 overexpressing carcinoma of the ovary.<sup>28</sup>

The functional basis of intrabody utility is closely linked to the ability of the scFv to interact with its target and prevent its maturation, thus limiting its contribution to the neoplastic phenotype. Thus, for optimal function an intrabody must be expressed in target cells at high level, achieve appropriate subcellular localization permitting interaction with its target, and maintain high affinity sufficient for effective capture of the corresponding oncoprotein. To this end, we have explored the utility of efficient gene delivery vehicles in the context of intrabody knockout strategies.<sup>27-30</sup> As well, our group, and others, have confirmed the key importance of employing heterologous cellular processing signals for achieving effective intrabody colocalization with target oncoproteins.<sup>16-24;30-32</sup>

In the present study we sought to determine if improvement in the affinity of the parent scFv could offer an additional means to improve the efficiency of intrabody-mediated oncoprotein knockout.

We have previously reported on the utilities of an anti-erbB-2 intrabody approach in the context of erbB-2 overexpressing tumor target.<sup>16-19</sup> In these studies, our intrabody was derived from the anti-erbB-2 scFv e23. In this regard, screening of a phage display library and site-directed mutagenesis

has been employed to obtain an anti-erbB-2 scFv such that this new derivative anti-erbB-2 scFv, C6.5, possesses an affinity for its target 1000 times greater than e23.<sup>33-35</sup> We thus employed this scFv to determine the degree to which affinity differential predicated utility for intrabody knockout approaches. Our results demonstrated that affinity enhancement of the parent scFv does not necessarily predict an improvement in an intrabody-mediated antineoplastic effects.

## **Material and Methods**

### ***Cell Lines***

The human ovarian cancer cell line SKOV3i.p1 was a generous gift from Janet Price (MD Anderson Cancer Center, Huston, TX); the prostate cell line DU145, human cervical cell line HeLa, human lung cancer cell line A549, and the breast cancer cell line MCF-7 were all obtained from ATCC (Rockville, MD) and maintained in DMEM/F12 (DU145, SKOV3.ip1), DMEM (HeLa) or RPMI (A549 & MCF-7) (Mediatech, Inc, Herndon, VA) supplemented with L-glutamine (300 ug/ml), penicillin (100 I.U./ml), streptomycin (25 ug/ml) and 10% fetal calf serum at 37°C in a humidified 5% carbon dioxide atmosphere.

### ***Plasmid construction and preparation***

The anti-erbB-2 scFv C6.5 and anti-erbB-2 scFv e23 were cloned into the plasmid pSTCF.KDEL, which contains an endoplasmic reticulum retaining

signal, giving rise to the plasmids pSTCF.C6.5 and pSTCF.e23. The plasmid pSTCF.LMP1 was obtained from a similar plasmid encoding the control anti-LMP1 scFv. The construction was as follows. The scFv expression plasmid pSTCF.KDEL was derived from pSecTagC (Invitrogen, San Diego, CA). This vector, derived from the plasmid pcDNA3, encodes an IgK leader sequence that targets encoded proteins into the secretory pathway, followed by a polylinker, an in-frame myc tag epitope, and a KDEL sequence. Expression is driven by the cytomegalovirus (CMV) promoter. In order to allow in-frame cloning of SfiI / NotI flanked scFvs from pCANTABS5E (Pharmacia Biotechnology) and other scFv phage display vectors, the pSecTagC vector was digested with SfiI and ApaI. The following complementary oligonucleotides were then annealed and ligated into the digested plasmid vector: 5' CGG CCG TTA ACG CGG CCG CCG GGC C 3' and 5' CGG CGG CCG CGT TAA CGG CCG GCT 3'. The resulting construct was verified by sequencing, and named pSTCF. To construct pSTCF.KDEL, the pSTCF plasmid was digested with NotI and ApaI. The following complementary oligonucleotides were then annealed into the digested plasmid vector: 5' GGC CGC CGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GAA GAA AAG ACG AAC TCT GAG GGG TTC CGC C 3' and 5' CTC ACA GAG TTC GTC TTT TTC TGA ATT CAG ATC CTC TTC TGA GCT GAG TTT TTG TTC GGC 3'. These oligonucleotides replaced the His tag present in the original



pSTCF construct with an internal myc tag and the ER retention sequence SEKDEL. Lastly, we cloned our e23 scFv and C6.5 scFv in this construct using SfiI and NotI restriction sites. The plasmid for the control gene pSTCF.LMP1 was provided by Dr. Alain Piche.

### ***Plasmid transfection***

All transfections were performed using the adenovirus/polylysine system (Ad/pL), as previously described.<sup>36</sup> This vector, consisting of a replication-incompetent adenovirus conjugated to poly-L-lysine, has been previously shown to achieve highly efficient transfection of SKOV3.ip1, DU145, A549 and HeLa cells. Briefly, 6 ug of plasmid DNA was conjugated with an optimized amount of AdpL vector and 4 ug of free poly-L-lysine, constituting one complex. Complexes were diluted in culture medium containing 2% FCS and added to cells in 6 well plates at a ratio of 1 complex per 1 million cells. After 1 hour, an equal volume of complete medium was added to the wells. Cells were then harvested and analyzed at various time points post-transfection.

For the study of cytotoxicity, a 2.5 ul fraction of a single AdpL complex was mixed with 100 ul of the appropriate 2% media and incubated with the cells for 1 hr. At that time, 100 ul of complete media was added. Cells were then incubated for 48-96 hr after transfection, an assayed as described below.

### ***Immunoblotting***

To assess the expression of protein by Western blot, cells were lysed for 30 minutes on ice with RIPA buffer (150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA and 50 mM Tris, pH7.4), by adding 300 ul per well in a six-well plate. Cells were then scraped, collected in microfuge tubes, and spun at 14,000 rpm for 15 minutes at 4°C. Supernatants were removed and protein concentration was assessed using the BioRad protein assay (BioRad, Hercules, CA) according to the instruction provided by the manufacturer. After mixture with 2X Gel Loading Buffer, 50 ug of total protein were separated on 12% SDS-PAGE gel (scFv) followed by transfer to PVDF membrane (BioRad). Membranes were probed with the anti-myc tag antibody 9E10 (Invitrogen, Carlsbad, CA) at a dilution of 1:2500, for scFv detection. HRP labeled goat-anti-mouse was used at a dilution of 1:5000 as secondary antibodies. Western blots were developed using the Renaissance reagent system (Dupont, Boston, MA).

### ***Immunoprecipitation***

To evaluate the interaction of the erbB-2 protein with the anti-erbB-2 scFv the whole cell lysate from DU145 cells transfected with the erbB-2 scFv plasmid was prepared as described above. It was then immunoprecipitated with a monoclonal human erbB-2 antiserum (Ab-3, Calbiochem). To this end, the

antibody was mixed with 500 ul of immunoprecipitation (IP) wash buffer (50 mM Hepes, pH7.4, 150 mM NaCl, 1 mM sodium vanadate, 1% Triton X-100, 10 % glycerol), 500 ul of Gold Buffer (2 mM Tris, 150 mM NaCl, gelatin, 0.1 % BSA, 0.5% Tween-20, 0.02% sodium azide), and 30 ul of protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), and rotated overnight at 4°C. The beads were then washed by brief centrifugation, followed by resuspension in 1 ml of IP wash buffer. This step was repeated 5 times. The beads were then resuspend with 500 ul of the cell lysate and rotated at 4°C for 2 hours. After washing as above, beads were resuspended in 100 ul of 1X SDS-PAGE sample buffer and placed on a 100°C heat block for 10 minutes. The samples were then evaluated by SDS-PAGE on a 12% gel, immunoblotted on to PVDF membranes (BioRad, Hercules, CA), and probed with anti-myc tag sera, as described above.

### ***Cellular cytotoxicity***

The effect of expression of the anti-erbB-2 scFv on cell growth and viability was determined employing the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation/Cytotoxic assay (Promega, Madison, WI). This MTS-based colorimetric assay measures the ability of viable cells to convert a tetrazolium salt to the formazan compound. To accomplish this analysis, cells were seeded into 96-well cell culture plates at a density of 5,000 cells/well.

Transfer of the scFvs-encoding plasmids pSTCF.e23, pSTCF.C6.5 and pSTCF.LMP1 was performed employing the Ad/pL system, as described above. A 2.5 ul fraction of a single Ad/pL complex was mixed with 100 ul of appropriate 2% media and incubated with the cells for 1 hr, at which point 100 ul of complete media was added. The plate was subsequently incubated for up to 96 hr. The Cell Titer 96 kit was then used according to the manufacturer's instructions. Percent viability was calculated by the following formula:  $[(\text{Infected cells-blank})/(\text{Uninfected cells-Blank})] \times 100$ .

## Results

Plasmids were derived for intracellular eucaryotic expression of the anti-erbB-2 scFvs e23 and C6.5. Plasmid elements include an IgK leader sequence, a minor terminal leader sequence, a carboxy-terminal myc epitope tag, and a KEDEL signal for the achievement of retention of the expressed protein in the endoplasmic reticulum (**Fig. 1**). Constructions were confirmed by digestion with restriction endonucleases and restriction analyses (data not shown). High efficiency transfection of this plasmid construct was achieved via adenovirus-polylysine conjugate (Ad/pL) employing described techniques.<sup>36</sup> Initial analyses sought to confirm that the e23 or C6.5 intrabody construct could achieve comparable levels of intracellular expression. Such an outcome would thus allow attribution of any differential biological effects to the affinity difference of the scFvs. The initial study employed the erbB-2

negative human cervical cell line HeLa. Transfection of the HeLa with Ad/pL conjugate containing the e23 plasmid pSTCF.e23 yielded a 35-kDa band when cell lysates were immunoblot analyzed with an anti-myc antibody (**Fig. 2A**). The appropriate size of the anti-erbB-2 intrabody was confirmed by comparison to the control plasmid pSTCF.LMP1. This construct encodes a scFv to a target irrelevant to the current analysis, the LMP1 protein of EBV. Of note, the plasmid pSTCF.C6.5 resulted in expression of an appropriate size protein in amounts equal to that achieved by transfection of pSTCF.e23. Thus, the plasmid-driven expression of the scFv e23 and C6.5 are associated with comparable levels of anti-erbB-2 intrabody.

We next sought to validate that the intracellular expression of the anti-erbB-2 scFv was capable of achieving interaction with that target. For this analysis, target erbB-2 negative HeLa, and positive DU-145, cell lines were transfected with the plasmid constructs as before. Cell lysate from transfected cells were then immunoprecipitated with anti-erbB-2 antibody and subjected to denaturing gel resolution followed by immunoblot with anti-myc antibody. In this study, both of the anti-erbB-2 scFvs were detected from transfection of the erbB-2 positive cell line DU-145 (**Fig. 2B**). This finding confirmed that both anti-erbB-2 antibodies possessed the capacity to recognize their cognate target in the context of intracellular expression. As control, similar analysis of the erbB-2 negative HeLa cell line did not exhibit

any retained intrabody, confirming that anti-erbB-2 specificity of the intracellularly expressed scFv. To this point, we had thus established that the anti-erbB-2 scFv were both capable of recognizing their cognate target in the intracellular context and were both associated with comparable level of intracellular expression of intrabodies.

On this basis, it was thus relevant to directly compare the efficacy of the intrabodies in achieving antineoplastic effects. In this regard, our previous studies have demonstrated that intracellular expression of anti-erbB-2 scFv can induce a direct cytotoxicity in erbB-2 expressing tumor cells.<sup>17-19</sup> We thus sought to directly compare the e23 and C6.5 intrabodies for this capacity in the context of the erbB-2 positive prostate carcinoma cell line DU-145. Cells were transfected with the conjugate Ad/pL containing either pSTCF.e23 or pSTCF.C6.5 and analysis carried out at different times post-transfection for viable cells number. In this study it could be seen that the e23 intrabody elicited significant cytotoxicity compared to Ad/pL conjugates containing the control plasmid pSTCF.LMP1 (**Fig. 3**). Of note, the C6.5 intrabody induced a level of cytotoxicity comparable to the e23 antibody. The level of plasmid delivered in this study was not sufficient to induce quantitative tumor cell cytotoxicity. On this basis, any differential cytotoxicity of the test intrabodies would have been apparent in this study. Thus, despite the differential target

affinity of the e23 and C6.5 scFvs, their derivative intrabodies were of comparable efficiency in inducing tumor cell cytotoxicity.

We compared the intrabodies in two additional ways. First, we have previously found that the level of erbB-2 in target cells is an important predictor of anti-erbB-2 intrabody-mediated cytotoxicity.<sup>17</sup> We thus carried out paired transduction of the e23 and C6.5 encoding plasmids in the context of target tumor cell lines expressing variable levels of erbB-2. As before, Ad/pL transfection of the plasmid was followed by analysis of the cell survival. In this study, there was no significant difference in intrabody-mediated cytotoxicity for the e23 and C6.5 intrabodies (**Fig. 4**). Thus, even in the context of variable level of the erbB-2 target, the differential affinity of the e23 and C6.5 scFvs did not predict differentiated antitumor efficacy of the derivative intrabody.

In addition, we explored the potency of the intrabodies in the context of a limiting dilution analysis. In this regard, our previous studies have demonstrated a threshold effect for intrabody-mediated cytotoxicity.<sup>37</sup> In these studies, it was shown that a requisite level of intrabody plus target was required to elicit tumor cell cytotoxicity. We thus hypothesized that the higher affinity of the C6.5 scFv for its target might be manifested if limiting levels of the intrabody were expressed. Target DU-145 cells were thus transfected with various levels of the plasmid pSTCF.e23 and pSTCF.C6.5. Analyses for

cytotoxicity were endeavored as before. Whereas a threshold effect could be noticed for cytotoxicity, this threshold levels did not differ for the test intrabodies (**Fig. 5**). It was thus apparent that differential efficacy of the intrabodies, manifested as dose-related potency, could not be attributable to the differential binding affinities of the parent scFvs.

Oncogene knockout represents an attractive cancer gene therapy approach whereby a highly targeted intervention may be achieved.<sup>2-6</sup> Based on this concept, a number of methods have been endeavored to achieve selective gene product abrogation as a means to revert the transformed phenotype. These interventions have included antisense and ribozymes designed to interrupt oncogene expression at the transcriptional level, as well as dominant negative molecules and intrabodies to achieve direct oncoprotein knockout.<sup>9-16</sup> The capacity of these agents to achieve a highly selective antitumor effect might be clinically advantageous. This hypothesis is currently being evaluated in human clinical trials.<sup>28</sup>

In this context, intrabodies offer a number of practical advantages, which have warranted their further study. Chief among these advantages is the facile means whereby anti-oncoprotein scFvs may be derived. Such methods have included genetic techniques of deriving cDNA directly from hybridomas<sup>21-25; 34</sup> as well as biopanning of phage scFv<sup>33,35</sup> libraries and others.<sup>38</sup> These methods have allowed direct isolation of scFv against



others.<sup>38</sup> These methods have allowed direct isolation of scFv against virtually any subcellular target protein. Thus, unlike antisense and ribozymes whereby specific recognition, and ablation, of target oncogenes sequences must be empirically determined, scFv may be reliably derived from accessible substrate systems for their employ as intrabodies.

The inhibitory effect of each scFv depends on achieving sufficiently high levels of expression in the appropriate intracellular localization and on retaining their binding affinity to the corresponding oncoprotein. In this regard, scFv may be directed to selected subcellular compartments via heterologous processing signals, and this capacity has allowed knockout of oncoproteins within the nucleus, cytosol, mitochondrial membrane, and the endoplasmic reticulum.<sup>21-24;31</sup> Intracellular expression of scFv, however, is a parameter that cannot be predicted *a priori*. On this basis, limited panels of derived scFv must be directly screened in the context of eucaryotic cells for determination of the optimal expression profile. Subsequent to the achievement of threshold expression at the appropriate locale, the intrabody is presumed to interact with its target based on affinity dynamics derived from the parental scFv.

In the current study we sought to determine if affinity enhancement in the parental scFv would augment the anti-neoplastic efficacy of the derivative intrabody. In this regard, various techniques have been employed to

improved scFv affinity for cognate targets.<sup>33-35</sup> Such efforts have largely been endeavored in the context of the comparison of recombinant immunotoxins, whereby the affinity of the antibody clearly correlated with tumor targeting, and with the overall therapeutic efficacy. In our studies, increasing the binding affinity of the scFv did not translate into an improvement in intrabody-mediated tumor cell cytotoxicity induction. A variety of considerations may be invoked to explain this result. First, it is not feasible to actually determine the affinity of the intrabody for its target in the intracellular context; it is plausible that the affinity differential of the e23 and C6.5 scFv was not maintained at its ER site of intrabody residence. Alternatively, the co-localization of the intrabody and target erbB-2 within the confinement of the ER may allow interactions of a magnitude whereby affinity augmentation is irrelevant. In either event, these studies serve to highlight the degree to which a direct extrapolation of the utilities of scFv to the intrabody context does not have universal validity.

In our human trial we have employed an anti-erbB-2 scFv for erbB-2 overexpressing carcinoma of the ovary.<sup>28</sup> These studies, as well as preclinical studies in murine model systems, have served to highlight gene delivery into tumor cells as the major practical limitation in realizing the full benefit of intrabody approaches.<sup>39</sup> On this basis, intrabodies may be considered in the context of various other mutations compensation

approaches, whereby direct tumor cell transduction is a requisite for anti-tumor action. As such, these various approaches require quantitative, or near-quantitative tumor cell transduction, to allow a therapeutic result. The recognition that no vector system can presently achieve this end thus belies the notion that intrabodies, or any mutation compensation approach, is currently rational for clinical cancer gene therapy applications. None-the-less, strategies to potentially improve biologic action, in the context of limited gene delivery, such as we have endeavored here, remain an area of legitimate pursuit to realize the conceptual benefits potentially offered by oncogene knockout approaches.

## KEY RESEARCH ACCOMPLISHMENTS

Specifically, we have demonstrated in this study that:

- I. An intracellular anti-cyclin-D1 single chain antibody targeted to the nucleus of the cell is functional.
- II. We can achieve downregulation of cyclin-D1 protein.
- III. Expression of the nuclear form of this intrabody mediates specific G0/G1 block of the cell cycle and S-phase delay in cyclin-D1 overexpressing breast cancer cells.
- IV. The basis of these effects is specifically due to the knockout of the cyclin-D1 axis, and
- V. Enhanced chemosensitivity to CDDP in cell treated with the nuclear and ER form of the anti-cyclin-D1 scFv.

## REPORTABLE OUTCOMES

- A. Ablation of cyclin-D1 via single-chain antibody achieves cell cycle arrest, phenotypic alternation, and chemosensitivity in breast cancer cell lines.

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- B. Anti-neoplastic effect of anti-erbB2 intrabody is not correlated with scFv affinity for its target.

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Departments of Pathology; Cell Biology and Surgery; and Department of Obstetric and Gynecology, University of Alabama at Birmingham, Birmingham, AL. Cancer Gene Therapy, In Press.

## **CONCLUSIONS**

We have established the feasibility of employing intrabodies to achieve functional knockout of the cyclin D1 oncoprotein in breast cancer cells. This novel genetic intervention represents an effective means of achieving targeted phenotypic alternations in breast cancer cells over expressing the cyclin D1 protein. On this basis, our approach defined herein may represent a novel therapeutic approach for carcinoma of the breast.

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Figure 1

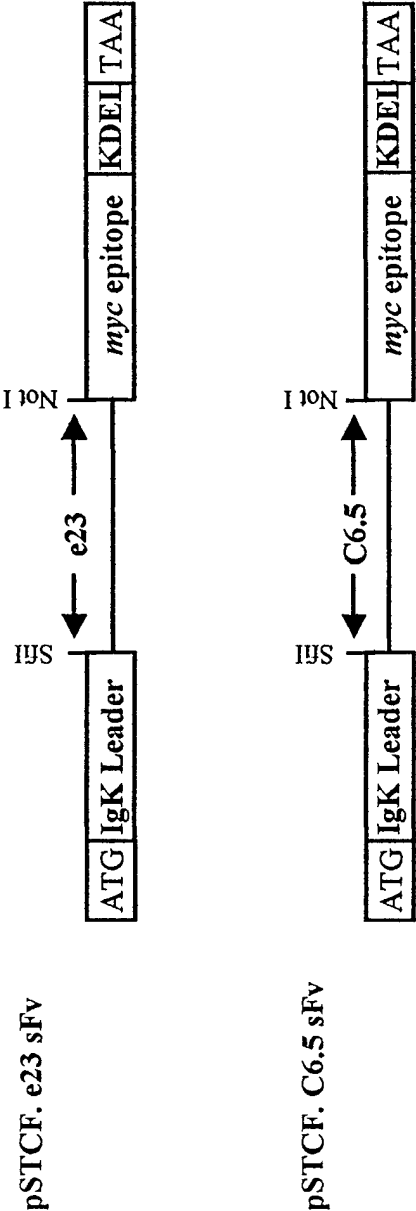


Figure 2A

A.

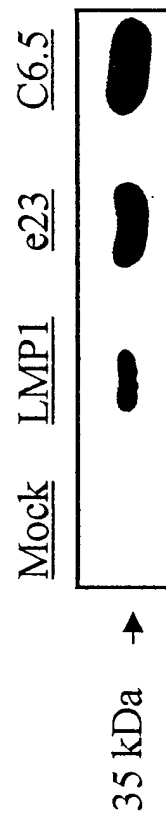


Figure 2B

B.

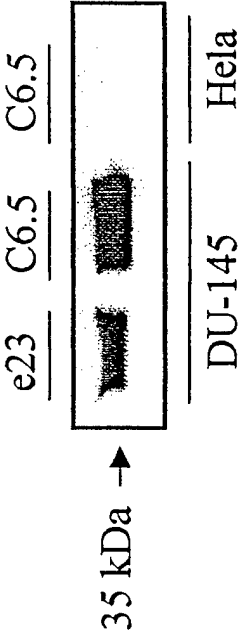


Figure 3

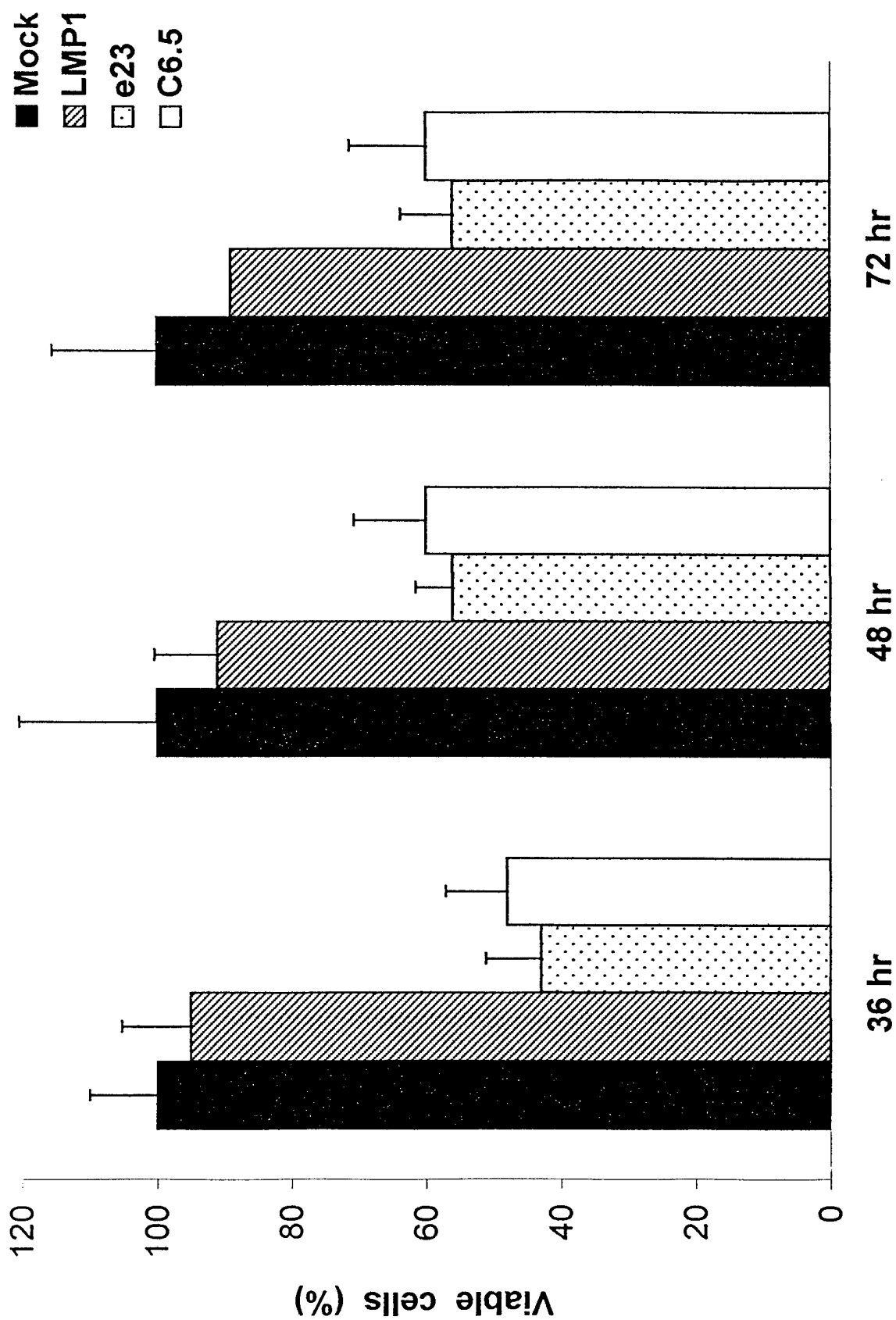




Figure 4

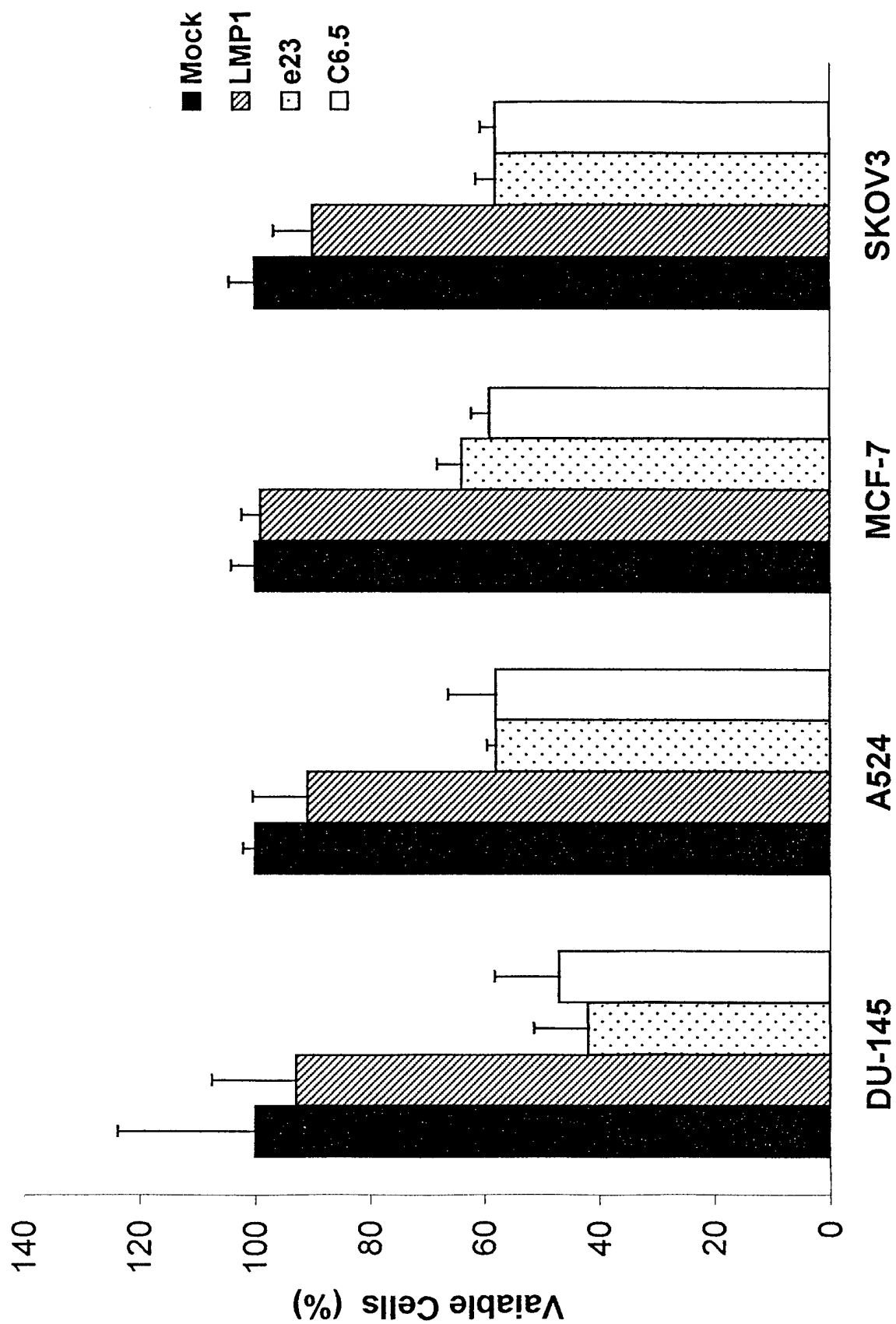
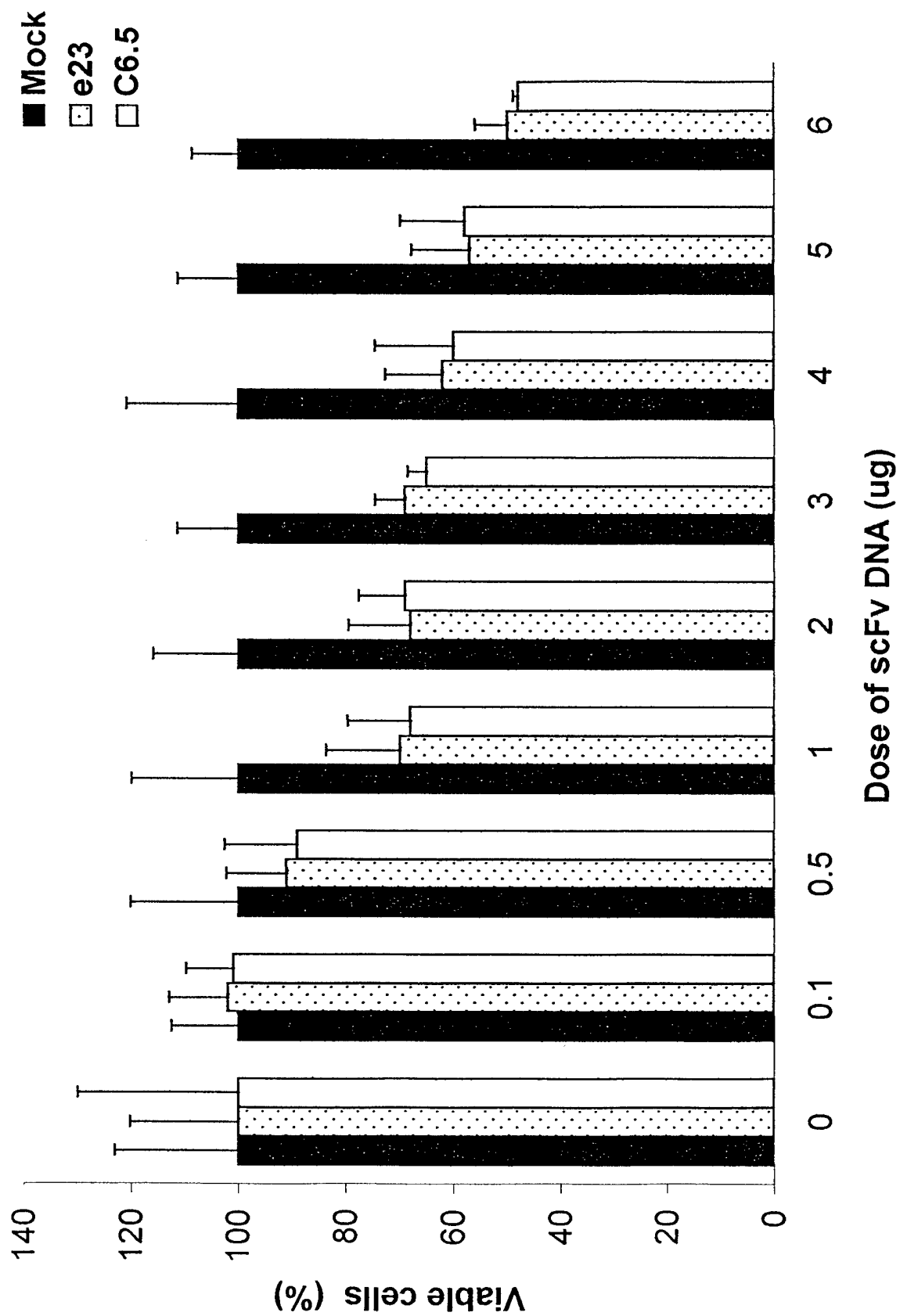


Figure 5



## Figure Legends

**Figure 1. Coding region of plasmids pSTCF.e23 and pSTCF.C6.5.** Open reading frame derived the scFv e23 and C6.5 were constructed into the parent plasmid pSecTag3. The coding region of the parent plasmid contains an ATG start site, an IgK leader sequence, the scFv cDNA, and a myc epitope tag and KDEL signal for retention in the endoplasmic reticulum (ER), followed by a TAA stop signal.

**Figure 2. Intracellular expression of anti-erbB-2 scFv. A.** Comparison of expression levels of e23 and C6.5 intrabody in erbB-2 negative cell line, HeLa. Cells were transfected with plasmids encoding LMP-1, e23, and C6.5 intrabodies, and cellular lysates analyzed for myc epitope. Mock represents untransfected control cells. Arrow indicates migration at 35 kDa. **B.** Evaluation of the target recognition of the e23 and C6.5 intrabodies. ErbB-2 positive DU-145 and erbB-2 negative HeLa were transfected with the intrabody. Cell lysates were immunoprecipitated by the anti-erbB-2 antibody, resolved by denaturing gel electrophoresis and then probed for myc by immunoblot. Arrow indicates migration of 35 kDa.

**Figure 3. intrabody-induced cytotoxicity in DU-145 tumor cells.** Transfection of DU-145 was carried out with plasmid encoding anti-erbB-2 intrabodies e23 or C6.5. Controls included untransfected cells (mock), or plasmid encoding irrelevant intrabody LMP-1. The DU-145 cells were analyzed for viability at 36, 48, and 72 hours post-

transfection. Results are indicated as percentage of viable cells compared to untransfected controls. Experiments were performed in triplicates and results are indicated as mean  $\pm$  SEM.

**Figure 4. Anti-erbB-2 intrabody-mediated cytotoxicity in various erbB-2 positive cell lines.** The indicated erbB-2 positive human carcinoma cell lines were transfected exactly as indicated in Figure 3. Analysis was performed at 48 hours for cell viability. Experiments were performed in triplicate and results are indicated as mean  $\pm$  SEM.

**Figure 5. Dose-response analysis of anti-erbB-2 intrabody-mediated cytotoxicity for DU-145 cells.** The erbB-2 positive human carcinoma cell line DU-145 was transfected with variable doses of the plasmids encoding e23 or C6.5 intrabodies. Analyses for cell viability were accomplished as before. Experiments were performed in triplicate and results are indicated as mean  $\pm$  SEM.

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### **Awards**

**Young Investigator Award, American Society of Gene Therapy, 1998.**

Title: "Ablation of cyclin-D1 via single chain antibody".

### **Publications**

1. Functional knock-out of c-myc by an intracellular anti-c-myc single-chain antibody. Keizo Kasono, Alain Piche, Jialing Xiang, Hyung Kim, Guadalupe Bilbao, Feng Johannning, Michael Nawrath, Karling Moelling, David T. Curiel. Submitted to Biochemical and Biophysical Research Communications.
2. Intracellular single chain antibodies for gene therapy. Guadalupe Bilbao, Jesus Gomez Navarro, Keizo Kasono, Juan Luis Contreras, David T. Curiel. In Methods in Molecular Medicine, Humana Press 1999, in press.
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4. Anti-neoplastic effect of anti-erbB-2 intrabody is not correlated with scFv affinity for its target. Waleed Arafat, Jesus Gomez Navarro, Jialing Xiang, Gene P. Siegal, Ronald D. Alvarez, David T. Curiel. In Cancer Gene Therapy 2000, in press.

### **Funded Personnel**

Guadalupe Bilbao

Waleed Arafat

## Abstract

As preliminary data:

1. Secretory single-chain antibodies as novel cytotoxic agents in the treatment of cancer cells. WO Arafat, J Xiang, JG Navarro, M Gonzalez, MN Barnes, RD Alvarez, GP Siegal, DT Curiel. 3<sup>rd</sup> Annual Meeting, American Society of Gene Therapy, Denver, Colorado, May 31- June 4, 2000. Poster
2. Secretory anti-erbB-2 single chain antibody as a novel cytotoxic agent in the treatment of ovarian carcinoma. WO Arafat, J Xiang, JG Navarro, P Mashasreshti, MN Barnes, RD Alvarez, GP Siegal, DT Curiel. 31<sup>st</sup> Annual Meeting, Society of Gynecologic Oncologists, San Francisco, California, February 5-9, 2000. Poster
3. Secretory anti-erbB-2 single-chain antibody as a novel cytotoxic agent in the treatment of erbB-2<sup>+</sup> cancer cells. WO Arafat, J Xiang, JG Navarro, M Gonzalez, B Liu, MN Barnes, RD Alvarez, GP Siegal, DT Curiel. 91<sup>st</sup> Annual Meeting, American Association for Cancer Research, San Francisco, California, April 1-5, 2000. Poster

## APPENDICES

1. Functional knock-out of c-myb by an intracellular anti-c-myb single-chain antibody. Keizo Kasono, Alain Piche, Jialing Xiang, Hyung Kim, Guadalupe Bilbao, Feng Johanning, Michael Nawrath, Karling Moelling, David T. Curiel. Submitted to Biochemical and Biophysical Research Communications.
2. Intracellular single chain antibodies for gene therapy. Guadalupe Bilbao, Jesus Gomez Navarro, Keizo Kasono, Juan Luis Contreras, David T. Curiel. In Methods in Molecular Medicine, Humana Press 1999, In Press.
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4. Anti-neoplastic effect of anti-erbB2 intrabody is not correlated with scFv affinity for its target. Waleed Arafat, Jesus Gomez Navarro, Jialing Xiang, Gene P. Siegal, Ronald D. Alvarez, David T. Curiel. Cancer Gene Therapy, In Press.

## Functional Knock-Out of c-myb by an Intracellular Anti-c-Myb Single-Chain Antibody

Keizo Kasono,\* Alain Piché,† Jialing Xiang,\* Hyung-Gyoon Kim,‡ Guadalupe Bilbao,\*  
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Aberrant expression of the c-myb proto-oncogene is a key factor in the development of the neoplastic phenotype in a variety of contexts. On this basis, it has been proposed that ablation of c-myb function might be an effective approach for therapy. To this end, we have employed an intracellular single-chain antibody (sFv) approach to achieve the functional knock-out of the c-Myb onco-protein. We derived an anti-c-Myb sFv, which was configured into eukaryotic expression plasmids. We confirmed the expression of the cytoplasmic and nuclear forms of the sFvs in the correct subcellular compartments by immunofluorescent staining. Importantly, the anti-c-Myb sFvs strongly inhibited the transactivation activity of c-Myb. Furthermore, cytotoxic effect of the sFv was observed only in the c-Myb positive cell line K562. These results suggest that anti-c-Myb sFv is a valuable tool for understanding the molecular mechanisms of c-myb induced transformation. In addition, this approach may have potential utility in the gene therapy for c-myb-dependent malignant diseases. © 1998 Academic Press

**Key Words:** single-chain antibody; c-myb; gene therapy; leukemia; transactivation.

The c-myb proto-oncogene is the cellular homologue of the viral oncogene, v-myb found in avian myeloblastosis virus (AMV) and E26 retroviruses (1,2). The product of the c-myb gene, c-Myb, is a transcriptional activator which plays an important regulatory role in cell proliferation and differentiation of hematopoietic cells (3-5). In this regard, high levels c-myb expression are detected in immature hematopoietic cells; during differentiation, the expression of c-myb is down-regulated

in the context of normal hematopoietic development (4-7).

In addition to its role in normal processes of differentiation, c-myb has been linked to the pathobiology of various neoplasms. Specifically, c-myb over-expression has been reported in human myelogenous leukemia (8-10) and lymphoreticular malignancies (8,11). In addition, it has recently been shown that c-myb over-expression is observed in malignant cells of nonhematopoietic origin, including colon cancer, melanoma, breast cancer, neuroblastoma and neuroepithelioma (8, 12-15).

Whereas c-myb overexpression has been linked to the neoplastic phenotype, its precise role in this context has not been determined. In this regard, we have developed a strategy for knockout of this oncoprotein by the intracellular single-chain antibody (sFv) approach. We have previously achieved down regulation and functional knock-out of oncoproteins such as erbB-2, Bcl-2, cyclin D1, Human papilloma virus 16 E7 (HPV 16 E7) and Epstein-Barr virus (EBV) latent membrane protein 1 (Lmp1) using the sFv technology (16-21). In this report, we demonstrate the functional knock-out of c-myb proto-oncogene product by an intracellularly expressed anti-c-Myb sFv. This technique may thus allow precise definition of the role of c-myb in neoplastic transformation. In addition, this approach may allow the development of the gene therapy strategies for c-myb-dependent malignancies.

### MATERIALS AND METHODS

**Cell lines.** K562 human chronic myelogenous leukemia cells and COS-1 african green monkey kidney cells were obtained from American Type Culture Collection (Rockville, MD). They were maintained in DMEM/F12 (COS-1) or RPMI1640 (K562) tissue culture media (Mediatech, Inc, Herndon, VA) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (25 µg/ml), and 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

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**Bacterial expression and purification of human c-Myb recombinant protein.** To express the amino-terminal half of c-Myb protein, the c-myb gene coding for amino acids 1-358 in the described plasmid, pET-8c/p42 (22) was cloned into BamHI and NotI site of PGEX4T-1, a GST-protein fusion system (Pharmacia Biotech Inc., Piscataway, NJ). According to the standard procedure, bacteria carrying the c-myb expression vector were grown and induced by isopropyl-B-D-thiogalactopyranoside (IPTG, Fisher Scientific, Fair Lawn, NJ). Resuspended bacteria were sonicated in the buffer (100 mM Tris-HCl pH 7.9 containing 300 mM NaCl and 1mg/ml of lysozyme). The GST-c-Myb fusion protein in the soluble fraction was then collected by glutathione sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech Inc.). After cleavage of the GST from the fusion protein by thrombin, reduced glutathione was added to release c-Myb protein from glutathione sepharose 4B. The purified c-Myb protein was then dialyzed using a Slide-A-Lyzer (pore size 10,000 MW, PIERCE, Rockford, IL) against 1.0 L of phosphate buffered saline (PBS) pH 7.4 with three buffer changes. The purified human c-Mybp42 was detected by immunoblotting using conditioned supernatant of hybridoma 4/14 secreting anti-c-Myb monoclonal antibody (MAb) (23).

**Construction of anti-c-Myb sFvs.** The 4/14 hybridoma cells, producing an anti-c-Myb MAb, were developed by H. Bading et. al. (23). The epitope recognized by the MAb is in the transactivating domain of human c-Myb, especially the sequence of amino acids from 123 to 134 (24). Messenger RNA was isolated from 4/14 mouse hybridoma cells using Poly (A) pure mRNA isolation kit (Ambion, Austin, TX). Anti-c-Myb sFvs were constructed using Recombinant Phage Antibody System Mouse sFv Module according to the manufacturer's instructions (Pharmacia Biotech). Briefly, the cDNAs of the variable heavy chain (VH) and light chain (VL) regions of anti-c-Myb IgG transcripts were synthesized using reverse transcriptase polymerase chain reaction (RT-PCR), following amplification of VH and VL using their respective primers. The sFvs were assembled by adding the linker (Gly<sub>4</sub>Ser)<sub>3</sub>. The sFv fragments were then digested with restriction endonucleases, SfiI and NotI, and cloned into the bacterial expression vector pCANTAB5E (Pharmacia Biotech), which contains an E-tag sequence (GAPVYPDPLEPR) at the C-terminus. The cloned sFvs were then directly transfected in *E. coli* HB2151 by the electroporation method. Screening of recombinant sFv clones was accomplished by the colony lift assay as described previously (25).

**Expression and functional assay of the sFv in a prokaryotic system.** Positive sFv clones from the colony lift assay were isolated, and expression of the sFv protein was induced by IPTG in 8 ml of 2 × YT media. To collect the periplasmic extract, the culture pellets were resuspended in 1 ml of cold PBS pH 7.4 containing 1 mM EDTA, and incubated on ice for 30 minutes with occasional vortexing. The mixture was centrifuged at 14,000 × g at 4°C, and the supernatant containing the soluble sFv protein was used for immunoblotting or the assay of binding ability of sFv by ELISA.

**Expression of anti-c-Myb sFv and c-Myb protein in eukaryotic cells.** The eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA) was used to construct a series of plasmids to achieve expression of the sFv or c-Myb protein in eukaryotic cells. Figure 1 shows the plasmids containing sFvs targeted to different subcellular compartments. The cytoplasmic sFv vector (pCyMybsFv) contains the anti-c-Myb sFv gene fused with c-Myc tag (EQKLISEEDLN). The nuclear sFv vector (pNuMybsFv) contains the SV40 nuclear localization signal sequences (TMPPKKKKRVGGAQPA) (26) plus the c-Myc tag at the C-terminal of the sFv. To achieve exogenous expression of the human c-Myb protein, the c-myb cDNA fragment isolated from pET-8c/p42, was cloned into pcDNA3 between BamHI and NotI site (pc-mybp42). Cells were transfected with the described plasmids by the adenovirus poly-L-lysine vector system (AdpL method), as previously described (16,27). At 48 h or the described time points, expression of the sFv or the c-Myb protein was determined by immunoblotting using anti-c-Myc tag polyclonal antibodies (A-14, Santa

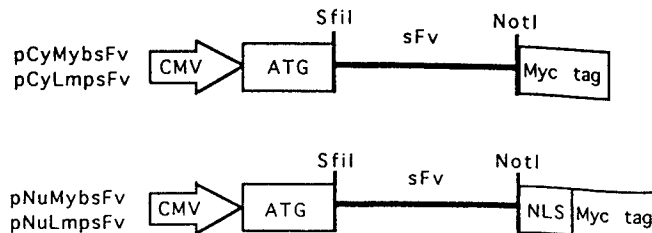


FIG. 1. Schema of the eukaryotic expression vectors of sFvs. All sFvs were cloned into pcDNA3 backbone plasmid. NLS: Nuclear localization signal.

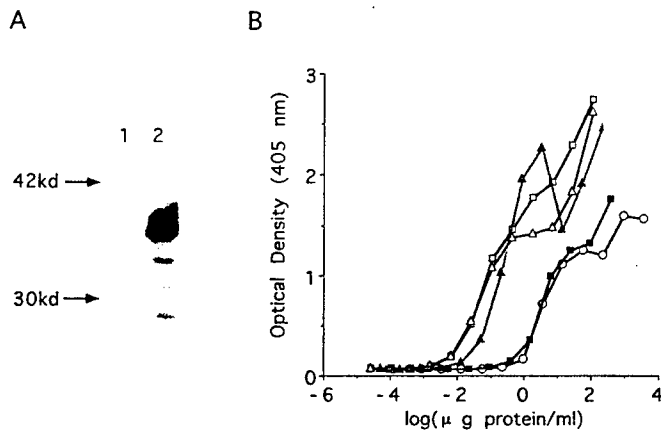
Cruz Biotechnology, CA) or conditioned medium from 4/14 hybridoma cells containing anti-c-Myb MAb, respectively.

**Immunoblot analysis.** For the detection of anti-c-Myb sFv protein, 30 µg of the cell lysate was separated on a 10% SDS-PAGE gel. After electrophoresis, protein was transferred onto a nitrocellulose membrane (BIO-RAD). An anti-E tag MAb (Pharmacia Biotech), at 1:1,000 for the prokaryotic system, or anti-c-Myc tag, at 1:3,000 for eukaryotic system, was added to the membrane as the primary antibody. After incubation, a HRP-conjugated goat anti-mouse, or anti-rabbit IgG (Jackson laboratories, Bar Harbor, ME), was added to the membrane and incubation was continued for an additional one hour. The immunoblot was developed by the Renaissance reagent system (Dupont, Boston, MA) according to the manufacturer's instructions.

**Immunofluorescence staining.** To detect the localization of the anti-c-Myb sFv *in situ*, COS-1 cells were plated on glass coverslips, and transfected with plasmids using the AdpL method. After 48 h, the cells were washed and fixed by PBS containing 4% paraformaldehyde. Then the cell membranes were permeabilized with PBS containing 0.2% TritonX100 (Sigma). An anti-c-Myc polyclonal antibody at 1:400 was added and incubated for 1 h at room temperature, followed by the incubation with fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-rabbit IgG antibody at 1:400 dilution for an additional 1 h. The stained cells were then evaluated by immunofluorescence microscopy.

**ELISA analysis.** Supernatant (100 µl) from the periplasmic extract of bacteria was added to the antigen-coated wells and incubated for 2 hour at room temperature. After treatment with wash buffer (PBS pH7.4 containing 0.1% Tween 20), 100 µl of anti-E tag antibody at 1:1000 was added to the plate, followed by a further 1 h incubation. After washing, HRP-cojugated anti-mouse IgG at 1:3000 was added to each wells. After the final washing, the color was developed with ABTS chromogen reagent (Sigma), and OD was read at 405 nm.

**Transcriptional assays.** To observe the inhibitory effect of anti-c-Myb sFv on the transcriptional activity of c-Myb, we employed a human c-myc promoter-luciferase fusion gene, pMyc-Luc (28) as a target of c-Myb. In this regard, it has been reported that c-Myb stimulates the human c-myc promoter, P1 and P2 (29). Co-transfection of plasmids, pMyc-Luc and pc-mybp42 into COS-1 cells showed stimulatory effect of c-Myb on the c-myc promoter. After establishing the transcriptional assay system, we also co-transfected pNuMybsFv or pCyMybsFv as a third plasmid. COS-1 cells were transfected with these plasmids by the AdpL method. Effector (pc-mybp42), reporter (pMyc-Luc) and inhibitor (pNuMybsFv or pCyMybsFv) plasmids were transfected in relative amounts of 34 : 1 : 67. Forty eight hours post-transfection, the cells were lysed in 100 µl of lysis buffer (Promega). 10 µl of each sample were subsequently mixed with 50 µl of luciferase assay reagent (Promega) according to the manufacturer's instructions, and duplicate determination of triplicate samples were assayed in a Berthhold luminometer. The luciferase activity was standardized by the amount of total protein.



**FIG. 2.** Expression and binding ability of an anti-c-Myb sFv expressed in *E. coli*. (A) Immunoblot analysis of the anti-c-Myb sFv expressed in *E. coli*. 10 l of periplasmic extract was separated by SDS-PAGE (12%) and sFv expression was determined by anti-E-tag antibody. HB2151 is the control periplasmic extract from non-transfected bacteria. (B) Binding ability of bacterial expressed sFv clones. ELISA was used to measure the binding activity of the periplasmic expressed anti-c-Myb sFv clones. Described concentrations of protein (100  $\mu$ l) from periplasmic extracts or supernatant of hybridoma 4/14 (○) were added to a 96-well plate coated with purified recombinant human c-Mybp42 (10g/ml). Clone 3 (□), clone 12 (△), clone 23 (■) and clone 33 (▲) were positive clones from colony lift assay.

**Stable transfections.** For analysis of phenotypic effect of the sFv on leukemia cells,  $4 \times 10^6$  K562 cells were transfected with 20  $\mu$ g of the linearized plasmids by electroporation. As a control, either linearized pcDNA3 or anti-Lmp1 sFv-containing vector was also employed for transfection. After 48 h of incubation in non-selective medium, the transfected cells were plated in 96 well plates at different concentrations, and the number of G418 resistant colonies were counted after 21 days of selection. For COS-1 cells, the cells (25,000 per well) were plated in 6 cm dishes and allowed to recover for 24 h before transfection. Cells were transfected using Lipofectamine (GibcoBRL) according to the manufacturer's recommendations. Three weeks later, the number of G418-resistant colonies were counted by crystal violet staining.

**Statistics.** Comparison of individual conditions were assessed using the Students' t test for equal means using StatView for Macintosh (SAS Institute Inc, San Francisco, CA).

## RESULTS

**Derivation of an anti-c-Myb sFv.** Several positive colonies were isolated by colony lift assay and expression of the anti-c-Myb sFv protein was confirmed by immunoblotting. The sFv protein is predicted to be composed of approximately 270 amino acids with an estimated molecular weight of 30 Kd. However, the position of the protein on the SDS-PAGE gel is slightly higher than the calculated molecular weight (Fig. 2A). Of note, this has also been observed in the context of other studies observing expression of sFvs in *E. coli* or mammalian cells (18,21). The binding activities of the engineered anti-c-Myb sFvs to the recombinant c-Myb protein were determined by ELISA assay (Fig. 2B).

Four of these sFv clones showed a dose-dependent binding affinity. Periplasmic extract of bacteria includes many types of proteins. Therefore, absolute binding affinities of these sFvs were not able to be calculated in this experiment. However, the optical densities achieved by sFv clones exceeded that exhibited by supernatant of original hybridoma cells secreting anti-c-Myb MAb. After repeating this experiments several times, we found that clone 23 had the lowest nonspecific binding affinity to the control protein, bovine serum albumin (BSA). For this reason, sFv clone 23 was used in subsequent experiments.

**Expression of anti-c-Myb sFvs in eukaryotic cells.** After validating the binding affinity of the derived anti-c-Myb sFvs expressed in bacteria, we constructed a series of plasmids to achieve the expression of the sFv in the eukaryotic cells. These plasmids were designed to localize the sFvs to specific subcellular compartments. COS-1 cells were transfected with pCyMybsFv or pNuMybsFv by the AdpL method, and stained with an anti-c-Myc antibody at 48 h post-transfection. Cytoplasmic sFv (pCyMybsFv) was expressed mainly in the cytosol. There was also some nuclear staining of intracellular sFv with this plasmid as well (Fig. 3A). The expression of the nuclear-targeted sFv (pNuMybsFv) was restricted to the nucleus (Fig. 3B). Thus, these studies confirmed that the anti-c-Myb sFvs were expressed in correct subcellular localizations in COS-1 cells.

**Time course of the expression of anti-c-Myb sFvs.** We also observed the time course of the expression of anti-c-Myb sFv. COS-1 cells were transfected with pCyMybsFv or pNuMybsFv by the AdpL method. After transfection, cell lysates were collected at various time points. The expression of the sFv protein was determined by immunoblot analysis. The expression of a cytosolic form of sFv was observed from 15 h to 160 h post-transfection (Fig. 4). The expression of a nuclear form of sFv was also detected from 15 h post-transfection. Although the expression level of a nuclear-targeted sFv was lower than that of cytoplasm-targeted sFv, peak of the expressions of both type of sFvs were observed around 90 to 120 h after transfection.

**Functional knock-out of c-Myb by anti-c-Myb sFvs.** We next determined the ability of the sFv to ablate the transactivation activity of c-Myb. To this end, we employed a human c-myc promoter-luciferase fusion gene. Previous studies showed that human c-myc promoter, P1 and P2 include c-Myb binding sites, and are strongly stimulated by human c-Mybp42 (29). In our experiments, we used the pMyc-Luc plasmid including P1 and P2 regions of human c-myc promoter fused with the luciferase gene (28). The c-Myb expression plasmid stimulated the luciferase activity several fold (Fig. 5, bar 2). Co-transfection of the COS-1 cells with

A



B

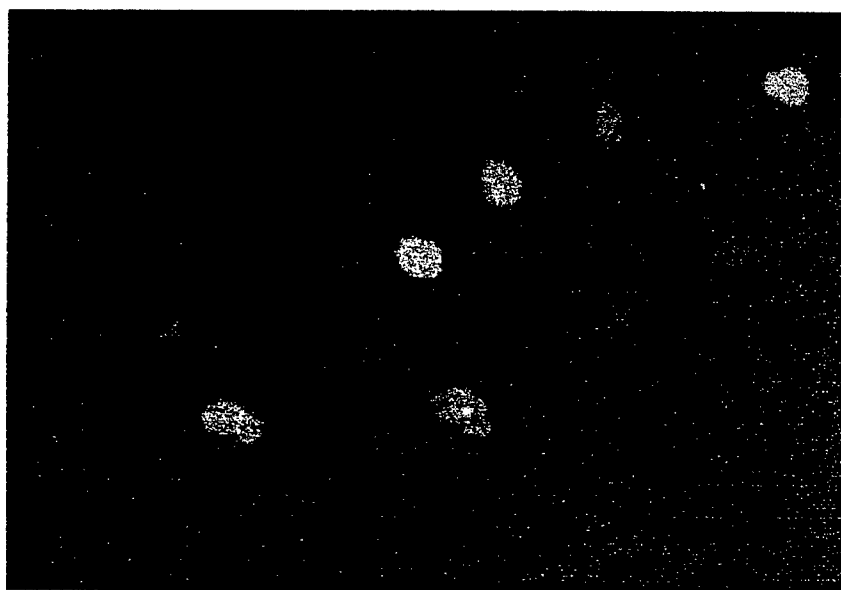


FIG. 3. Immunofluorescent staining of cytoplasmic- and nuclear-targeted anti-c-Myb sFvs. (A) COS-1 cells were transfected with cytoplasmic form of anti-c-Myb sFv, pCyMybsFv by the AdpL method. After 48h, the sFv was detected by anti-c-Myc-tag antibody and FITC-labeled anti-rabbit IgG. (B) COS-1 cells were transfected with nuclear form of anti-c-Myb sFv, pNuMybsFv.

pNuMybsFv, pc-mybp42 and pMyc-Luc showed that the nuclear form of the anti-c-Myb sFv inhibited the transactivating activity of c-Myb to the basal level (Fig. 5, bar 4). The cytoplasmic form of the anti-c-Myb sFv has the same effect on the function of c-Myb (Fig. 5, bar 6). Importantly the control plasmid pNuLmpsFv and pCyLmpsFv showed no significant inhibitory effect on the activity of c-Myb (Fig. 5, bar 3 and 5). Control sFvs, pNuLmpsFv and pCyLmpsFv did not show the inhibitory effect on the activity of c-Myb (Fig. 5, bar 3 and 5). These results suggest that nuclear and cytoplasmic

forms of anti-c-Myb sFv specifically blocked the function of c-Myb.

*Effect of anti-c-Myb sFv on derivation of stable clone in c-myb-positive and -negative cell lines.* To evaluate the effects of the anti-c-Myb sFv, we attempted to derive stable clones from several cell lines. A differential in stable clone derivation is a common assay used to indicate specific functional effects of the sFv in cell lines. The plasmid pNuMybsFv, encoding nuclear-targeted anti-c-Myb sFv, and control plasmids pcDNA3 or pNuLmpsFv were used in this experiments. Trans-

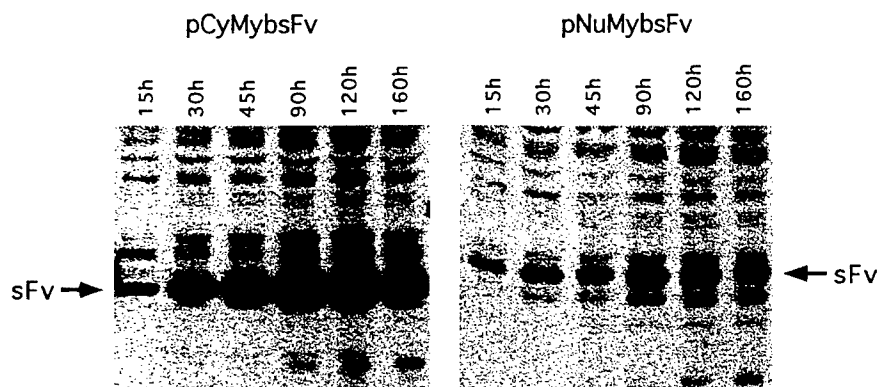


FIG. 4. Time course of the expression of anti-c-Myb sFvs. COS-1 cells were transfected with pCyMybsFv (left) or pNuMybsFv (right) by the AdpL method. At the described time points, cell lysates were separated on a 10% SDS-PAGE gel.

fection of the anti-c-Myb sFv did not elicit suppression of colony formation in the c-myb-negative cell line, COS-1 (Table 1). In contrast, there was a 60% reduction of colony formation in the c-myb-positive K562

cells transfected with anti-c-Myb sFv. Thus, the effects of the anti-c-Myb sFv appeared to be dependent upon the expression of c-myb in the target cells. Nonetheless, the control sFv plasmid, pNuLmpsFv, also caused a level of suppression of colony formation of the cell lines. The basis for this effect may reflect the antibody cross-reactivity with alternate cellular target, or may represent a nonspecific effect of the intracellular antibody on normal cellular physiology.

## DISCUSSION

In this report, we demonstrate the functional knock-out of c-Myb proto-oncoprotein by an intracellular anti-c-Myb single-chain antibody. We observed the anti-c-Myb sFvs were expressed in mammalian cells and could be targeted to subcellular compartments. Without any signal sequence, the sFv proteins were translated in cytosol and distributed in a diffuse pattern. In our experiments, cytoplasm-targeted sFv was detected both in the cytosol and nucleus. This is probably because the small sized proteins easily enter the nucleus through the pores in the nuclear membrane without nuclear leader signal peptide. For example, green flu-

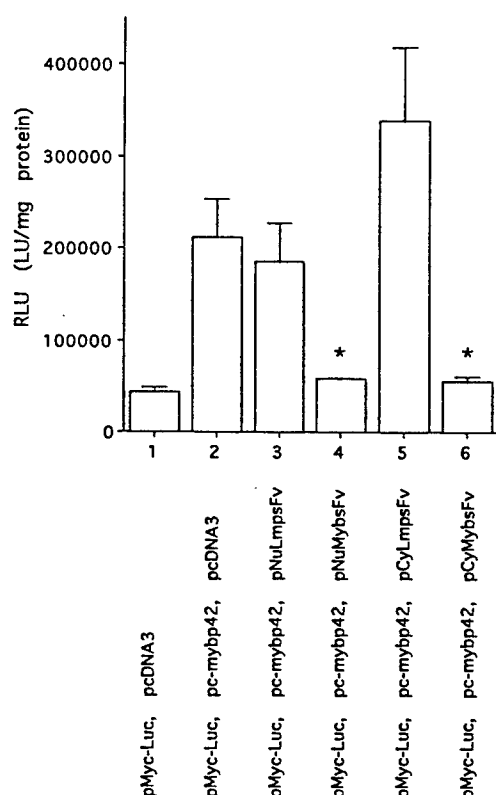


FIG. 5. Transcriptional assay of the c-myc promoter. Lane 1 showed the basal c-myc promoter activity. Subconfluent COS-1 cells in a 12-well plate were transfected with myc promoter-luciferase fusion plasmid, pMyc-Luc (0.002 $\mu$ g) and mock plasmid, pcDNA3 (0.2 $\mu$ g). After 48 h, luciferase activity was measured. COS-1 cells were transfected with pMyc-Luc (0.002 $\mu$ g), c-Myb expression plasmid, pc-mybp42 (0.067 $\mu$ g) and mock plasmid (0.133 $\mu$ g) (bar 2). COS-1 cells were transfected with pMycLuc, pc-mybp42, and pNuLmpsFv (bar 3), pNuMybsFv (bar 4), pCyLmpsFv (bar 5) or pCyMybsFv (bar 6). \*  $P < 0.01$ , bar 2 vs bar 4 or bar 6.

TABLE I  
Effect of Anti-c-Myb sFv on Derivation of Stable Clones

Cell line	Number of G418-resistant colonies		
	pcDNA3	pNuLmpsFv	pNuMybsFv
COS-1	347 $\pm$ 51	305 $\pm$ 20	369 $\pm$ 48
K562	35 $\pm$ 3.8	23 $\pm$ 2.2	15 $\pm$ 3.2*

Note. Cell lines were transfected with either the pcDNA3, pNuLmpsFv or pNuMybsFv using Lipofectamine (COS-1) or electroporation (K562). At 48 h, G418 was added to the media. At 21 days after transfection, colony numbers were evaluated. Results are from four separate experiments and are expressed as mean  $\pm$  SD. \* $P < 0.01$ , pcDNA3 vs pNuMybsFv.

orescent protein which has similar molecular weight as sFv doesn't include a nuclear leader signal, but it can be detected both in the cytosol and the nucleus (data not shown). We observed the expression of the nuclear form of the sFv was restricted to the nucleus. SV-40 nuclear leader peptide has strong activity to target the protein to the nucleus (26). Compared to the cytoplasm-targeted sFv, the expression levels of nucleus-targeted sFv were low. However, the expression levels of the nuclear oncoproteins in malignant cells are also very low. In some malignant cells, only Northern blot analysis could detect the expression of c-myb (30). Importantly, the biological effects of antisense oligodeoxynucleotides on c-myb mRNA positive cells suggest that low level of c-Myb can have a strong effect on the phenotypic change of the cells (30, 31). Therefore, a small amount of expressed sFv may be sufficient to ablate the function of the nuclear oncoprotein. In our experiments, anti-c-Myb sFvs, pNuMybsFv and pCyMybsFv didn't decrease the expression level of c-Myb protein (data not shown). Usually, ER-targeted sFvs, anti-ErbB2, anti-Lmp1 and anti-Bcl-2 sFvs in our laboratory inhibit the expression of target protein in the cells (18,21). However, the binding of cytoplasmic form of sFv to the target protein was not necessary for down regulation of the expression of the target protein (32). Therefore, the cytosol- and nuclear-targeted anti-c-Myb sFv specifically blocked the function of c-Myb. In our experiment, both proteins, sFv and c-Myb were controlled under the CMV promotor. It required double amount of the pNuMybsFv or pCyMybsFv to totally block the effect of pc-mybp42 expressing c-Myb.

To detect the phenotypic effect of the sFv on malignant cells, we assayed the colony formation of cells transfected by the sFv. Reduced number of colonies derived from cells transfected by anti-c-Myb sFv compared to control suggests a cytotoxic effect of the sFv on the leukemia cells expressing c-myb. Consistent with this observation, c-myb negative cells did not show any reduction of colony formation by anti-c-Myb sFv. The results suggested that anti-c-Myb sFv has specific cytotoxic effect on leukemia cells expressing c-myb. In our experiment, anti-Lmp1 sFv also slightly reduced the number of clones especially in K562 cells. The parental Mab of anti-Lmp1 sFv, S12, recognizes the cytosolic domain of EBV derived TNF receptor family protein, Lmp1 (33,34). Previous study have shown that S12 Mab reacted with some EBV-negative normal tissues (35). In addition, the expression level of anti-Lmp1 sFv was much higher than that of anti-c-Myb sFv (data not shown). Therefore, it is possible that the control sFv has some cross reactivity with other target protein or proteins. Or the effects of the control sFv may reflect a nonspecific effect of the intracellular sFv on normal cellular physiology.

On the basis of our achievement, inhibition of the function of c-Myb will enable a more direct method of investigation of c-myb function. Until now, we could only use the over expression of exogenous c-Myb to approach the functional analysis of the oncoprotein. Although c-myb antisense oligodeoxynucleotides showed an inhibitory effect on the growth of malignant cells in previous reports (36,37), there is no direct evidence of functional knockout of c-Myb by the antisense oligodeoxynucleotides. We are currently evaluating the effect of the anti-c-Myb sFv on malignant cells expressing c-myb which may help a strategy for the gene therapy of leukemia and other c-myb-dependent malignancies.

## ACKNOWLEDGMENTS

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## **Intracellular Single-Chain Antibodies for Gene Therapy**

**Guadalupe Bilbao, Jesus Gomez-Navarro, Keizo Kazano,  
Juan Luis Contreras, and David T. Curiel**

### **1. Introduction**

The delineation of the molecular basis of cancer in general, allows for the possibility of specific intervention at the molecular level for therapeutic purposes. To this end, three main approaches have been developed: mutation compensation, molecular chemotherapy, and genetic immunopotential. The strategy of mutation compensation aims to correct the specific genetic defects in cancer cells. Such correction is accomplished by either ablation of oncogenic products, replacement of cellular tumor suppressor genes, or interference with dysregulated signal transduction pathways. A second strategy is molecular chemotherapy, which aims to increase the specificity of drug delivery or to increase tolerance to standard chemotherapeutic regimens. A third strategy, genetic immunotherapy, aims to augment the specificity and/or the magnitude of the normal immune response to tumors. For each of these conceptual approaches, human clinical protocols have entered Phase I clinical trials to assess dose escalation, safety, and toxicity issues.

The genetic lesions etiologic of malignant transformation may be thought of as a critical compilation of two general types: aberrant expression of "dominant" oncogenes or loss of expression of "tumor suppressor" genes. Gene therapy strategies have been proposed to achieve correction of each of these lesions. For approaching the loss of function of a tumor suppressor gene, the logical intervention is replacement of the deficient function with a wild-type tumor suppressor gene counterpart. The dysregulation of oncogenes by mutation, gene amplification, gene rearrangement, or overexpression

contributes the aberrant expression of the corresponding gene product that elicits the associated neoplastic transformation. One approach to gene therapy for cancer is to "knock out" dominant oncogenes and thereby reduce the tumor's growth or invasive potential. Inhibition or ablation of oncogenic function can be attempted at three levels. First, the translation of the oncogene can be targeted. This strategy involves the use of the most universally employed methodology the "antisense" molecules to sequester oncogene mRNA (1-4). However, despite the fact that antisense inhibition can be demonstrated in many contexts, targeting the uptake of oligonucleotides into cells, the stability of the antisense molecules, and pharmacokinetic considerations within animals, have greatly limited translation of this approach into human clinical trials (5-6). Second, the function of the gene product can be targeted. This approach uses polypeptides containing dominant interfering mutations to downregulate signal transduction in tumor cells. Third, the nascent oncogenic protein can be prevented from reaching its proper intracellular location. This approach uses intracellular single-chain Fv molecules (scFv) to preempt the cellular localization machinery and sequester proteins inside the cell. In this regard, single-chain immunoglobulin (scFv) molecules retain the antigen-binding specificity of the immunoglobulin from which they were derived, however, they lack other functional domains characterizing the parent molecule. The basis of constructing scFvs has been established. Pastan et al. have developed methods to derive cDNAs which encode the variable regions of specific immunoglobins (7-8). Specifically, a single-chain antibody (scFv) gene is derived which contains the coding sequences for variable regions from the heavy chain ( $V_H$ ) and the light chain ( $V_L$ ) of the immunoglobulin separated by a short linker of hydrophilic amino acids (see Fig. 1). The resultant recombinant molecule, when expressed in prokaryotic systems, is a single-chain antibody (scFv) which retains the antigen recognition and binding profile of the parental antibody (9-11). The development of recombinant immunotoxins employing scFv moieties achieves cell-specific binding of the toxin to the exterior of the target cell, allowing receptor-mediated endocytosis to accomplish toxin internalization. A variety of strategies employing the recombinant scFv-directed immunotoxins have been developed by a number of investigators (7,8,12-15). In addition, it has recently been shown that scFv molecules may be expressed intracellularly in eukaryotic cells by gene transfer of scFv cDNAs. The encoded scFv may be expressed in the target cell and localized to specific, targeted subcellular compartments by appropriate signal molecules. Importantly, these intracellular scFvs may recognize and bind antigen within the target cell. Target for the intracellular antibody knockout method have included viral antigens in the context of HIV infection (phase I clinical trial), transformed oncoprotein like erbB2 and cRas, and tumor associate antigens like CEA (16-18). In this regard, our



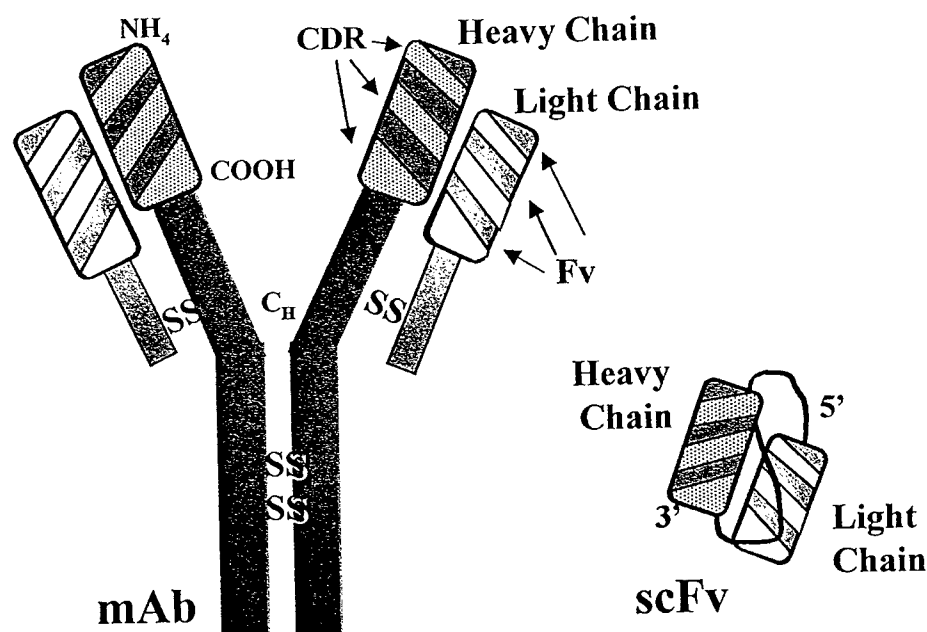


Fig. 1. Antibody structure, showing the heavy and light chain of an immunoglobulin. S-S denotes inter- or intramolecular disulfide bonds. The CDR residues, which form the antibody-binding site, are shown in dark in the variable regions. Single-chain Fv with VH and VL regions joined by a linker (black line).

group has recently exploited this technology to develop an anti-erbB-2 scFv that down-modulates the erbB-2 oncoprotein in erbB-2 overexpressing tumor cells (19–26). This method of genetic intervention achieved diverse antineoplastic effects specifically in tumor cells overexpressing the targeted oncoprotein. Importantly, this approach to human carcinoma of the ovary based on the anti-erbB-2 scFv strategy is now approved for a Phase I clinical trial (<http://www.nih.gov/od/orda/protocol.htm>).

This chapter will review in detail practical procedures to generate a single-chain intracellular antibody (see Fig. 2). Most of the methods we employ in our lab utilize commercially available kits for convenience. We will emphasize in this review the different steps in our protocol that we have employed to develop scFvs to a variety of target proteins. The Recombinant Phage Antibody System (RPAS) from Pharmacia (Uppsala, Sweden), is based on a phage-display technology where fragments of antibodies are expressed as fusion with gene-3 protein and displayed on the tips of M13 phage. Once antigen-positive clones have been identified by phage rescue, they are used to infect a nonsuppressor strain of *E. coli* HB2151 for the purpose of producing soluble

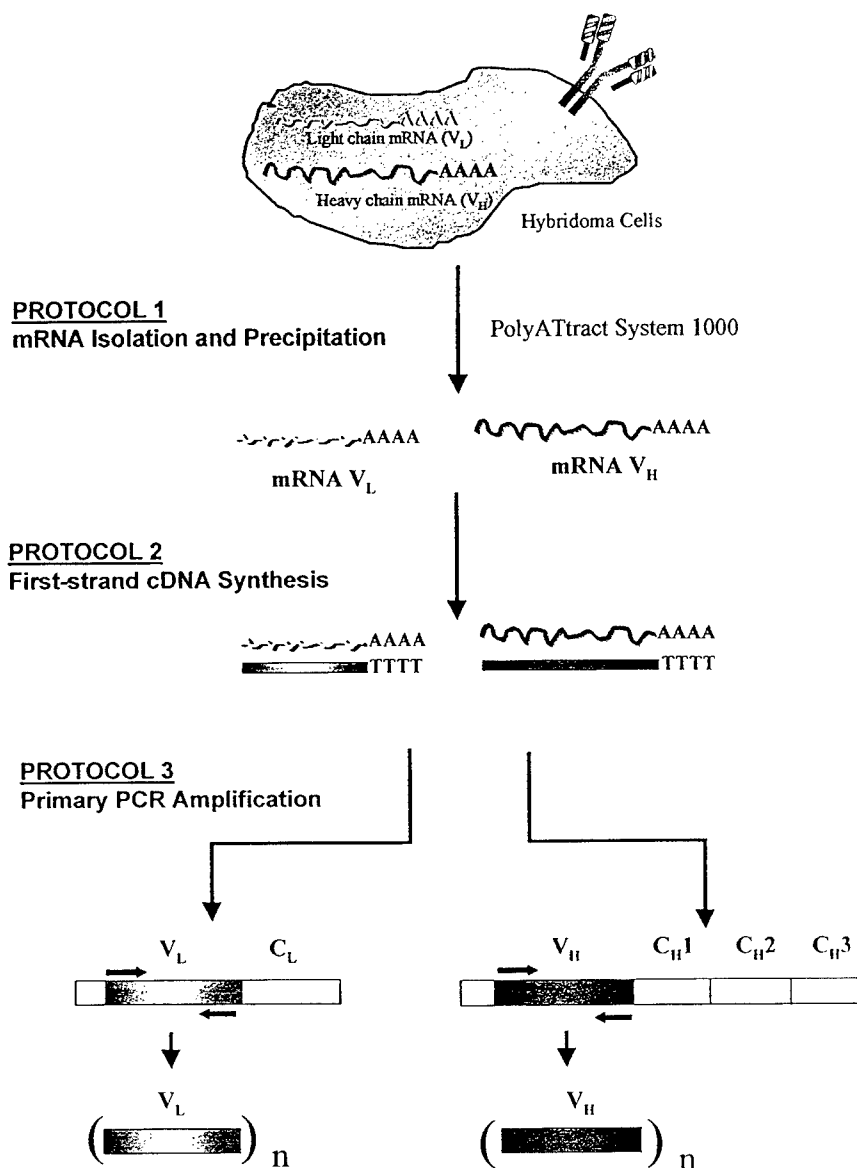
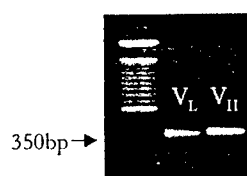


Fig. 2. Construction and screening of a single-chain antibody library.

antibodies. In our laboratory, we used a modified colony lift technology to identified antigen-positives clones directly into the nonsuppressor HB2151 *E. coli*. This modification in the expression module is a very simple way of screening your recombinant single-chain antibodies, saving time-consuming steps.

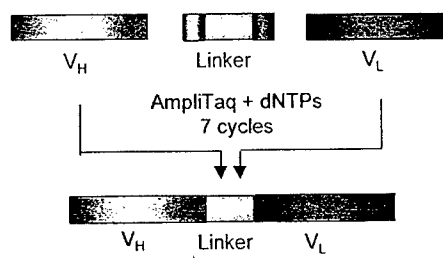
**PROTOCOL 4 and 5**

**Gel Analysis, Purification and quantification of Primary V<sub>H</sub> and V<sub>L</sub> PCR Products**



**PROTOCOL 6**

**Assembly And Fill In Reactions**



**PROTOCOL 7**

**Second PCR Amplification and Purification**

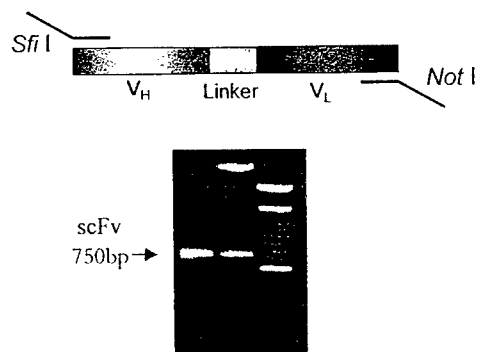


Fig. 2. (continued).

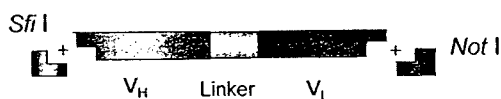
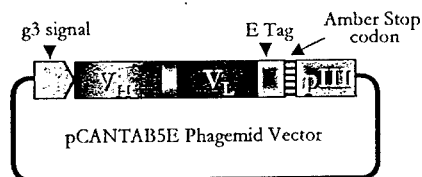
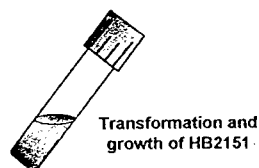
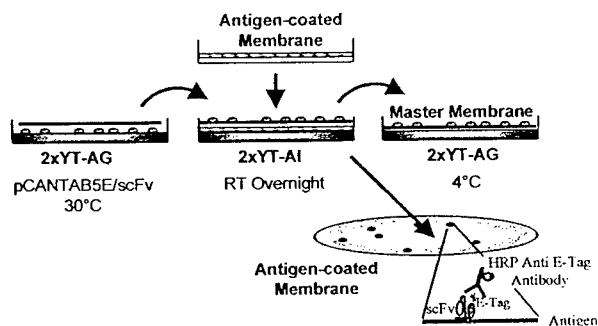
**PROTOCOL 8****Restriction Digestion and Purification****PROTOCOL 9****Ligation of the scFv cDNA into the Phagemid Plasmid pCANTAB5E****PROTOCOL 10****Transformation****PROTOCOL 11****Modified Colony Lift Assay**

Fig. 2. (continued).

**1. Materials**

All water used in these protocols should be sterile, deionized, and distilled.  
 All reagents, plastic and glassware should be sterile.

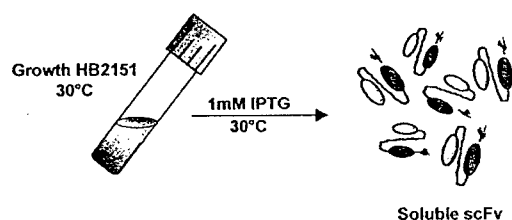
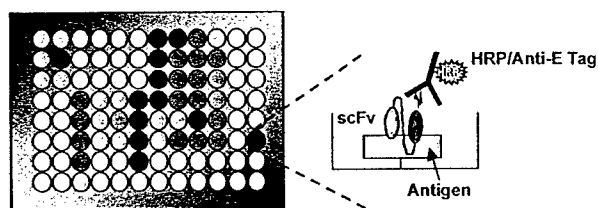
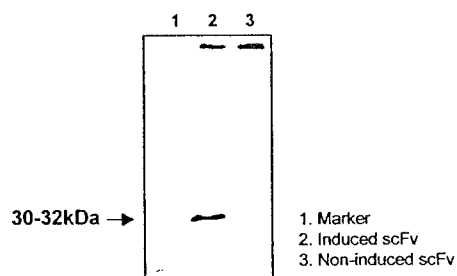
**PROTOCOL 12****Production of Soluble Antibodies (Periplasmic Extract)****PROTOCOL 13****Detection and Binding Specificity of E-Tagged scFvs****PROTOCOL 14****Western Blot Analysis of E-Tagged scFvs**

Fig. 2. (continued).

**2.1. Kits**

1. mRNA Purification PolyATtract 1000 Kit (Promega, Madison, WI, cat. # Z5420). Store at 4°C.
2. First-Strand cDNA Synthesis Kit (Pharmacia Biotech cat. # 27-9261-01). Store at -20°C.

3. Mouse scFv Module Recombinant Phage Antibody System (Pharmacia Biotech cat. # 27-9400-01). Store at  $-20^{\circ}\text{C}$ .
4. Mouse Ig-Prime Kit System (Novagen, Madison, WI, cat. # 70082-3). Store at  $-20^{\circ}\text{C}$ .
5. QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA, cat. # 28704). Store at room temperature.
6. pShooter Mammalian Expression Vector collection (Invitrogen, San Diego, CA, pCMV/myc/nuc cat. # V821-20, pCMV/myc/mito cat. # V822-20, pCMV/myc/cyto cat. # V820-20). Store at  $-20^{\circ}\text{C}$

## 2.2. Reagents

1.  $\beta$ -Mercaptoethanol: is toxic, dispense in a fume hood and wear appropriate personal protective equipment. Store at  $4^{\circ}\text{C}$ .
2. RNase Zap (Ambion, Austin, TX, cat. # 9780 ). Store at room temperature.
3. Isopropylthio- $\beta$ -D-galactopyranoside (IPTG). Store at  $-20^{\circ}\text{C}$ .
4. Ethanol. Store at room temperature.
5. Mineral oil: Light Mineral Oil (Fisher, Pittsburgh, PA, cat. # 0121-1). Store at room temperature.
6. AmpliTaq<sup>®</sup> DNA Polymerase from (Perkin-Elmer, Norwalk, CT, cat. # N808-0038). Store at  $-20^{\circ}\text{C}$ .
7. Ultrapure DNA grade Agarose (Bio-Rad, Richmond, CA, cat. # 162-0133). Store at room temperature.
8. 0.5X and 1X TAE Buffer: 50X TAE 121g Tris, 50 mL 0.5M EDTA pH 8.00, 28.55 mL glacial acetic acid, QS to 500 mL with ddH<sub>2</sub>O. Store at room temperature.
9. RNase-Free Solutions: Add diethylpyrocarbonate [DEPC] (Sigma, St. Louis, MO, cat. # D-5758) to solution to a concentration of 0.05% (i.e., add 0.5 mL per liter of solution); shake well, incubate several hours to overnight at  $37^{\circ}\text{C}$  of  $42^{\circ}\text{C}$ ; autoclave at least 45 min, or until DEPC scent is gone. Store at room temperature.
10. 0.5M EDTA: 186.1 g EDTA, 20 g NaOH pellets, pH to 8.00 with NaOH, QS to 1 L with ddH<sub>2</sub>O. Store at room temperature.
11. 100 Base-Pair ladder (Life Technologies, Gaithersburg, MD, cat. # 15-628-019). Store at  $-20^{\circ}\text{C}$ .
12. Ethidium bromide 100 mg/mL (Bio-Rad cat. # 161-0433). Store at room temperature.
13. 6X Loading Dye: 30% glycerol and 0.25% bromophenol blue in 10 mM TE, pH 7.6. Store at room temperature.
14. dNTP Mix (100 mM each of dATP, dCTP, dGTP, dTTP in sterile water). Store at  $-20^{\circ}\text{C}$ .
15. 25 mM MgCl<sub>2</sub> sterile. Store at room temperature.
16. 10X PCR buffer (Perkin-Elmer cat. # N808-0038). Store at  $-20^{\circ}\text{C}$ .
17. TE Buffer: 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Store at room temperature.

18. *Sfi* I (Boehringer Mannheim, Mannheim, Germany, cat. # 1288-016). Store at  $-20^{\circ}\text{C}$ .
19. *Not* I (Boehringer Mannheim cat. # 1014-706). Store at  $-20^{\circ}\text{C}$ .
20. 3 M NaCl: 175.35 gr of NaCl, QS to 1 L with ddH<sub>2</sub>O. Store at room temperature.
21. Phenol:chloroform:isoamyl alcohol (25:24:1), equilibrated against TE (Amresco, Solon, OH, cat. # 0883). Store at  $-20^{\circ}\text{C}$ .
22. Phosphate Buffered Saline (PBS): 0.2 g KCl, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, 8 g NaCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, QS to 1 L with ddH<sub>2</sub>O. Store at room temperature.
23. T4 DNA Ligase (Boehringer Mannheim cat. # 481-220). Store at  $-20^{\circ}\text{C}$ .
24. 30% H<sub>2</sub>O<sub>2</sub> (any company). Store at  $4^{\circ}\text{C}$ .
25. 2M glucose. Do not autoclave, filter sterilize. Store at room temperature.
26. HRP/Anti-E Tag Conjugate (Pharmacia cat. # 27-9412-01). Store at  $-20^{\circ}\text{C}$ .
27. Chloro-1-Naphthol (4C1N) tablets (30mg) (Sigma cat. # C-6788). Store tablets at  $-20^{\circ}\text{C}$ . Protect from heat, light and moisture. Allow to reach room temperature before use.
28. Triethanolamine saline pH 7.5: Add 7.5 gr of NaCl, 2.8 mL of triethanolamine (Sigma cat. # T-1377), and 17 mL of 1M HCl to approx 800 mL of dH<sub>2</sub>O. Adjust the pH to 7.5 if necessary and QS to 1 L with dH<sub>2</sub>O. Store at room temperature.
29. ABTS (2', 2'-Azino-Bis[3-Ethylbenzthiazoline-6-Sulphonic Acid] Diammonium) add the following to a 500 mL: 450 mL of 0.05M citric acid pH4.0, and 100mg of ABTS. Filter sterilize the 1X ABTS stock solution and store at  $4^{\circ}\text{C}$  until needed.
30. 40% Acrylamide/Bis Solution 37.5:1 (Bio-Rad cat. # 161-0148). Store at  $4^{\circ}\text{C}$ .
31. 10% SDS (Life Technologies, cat. # 24730-012). Store at room temperature.
32. Ammonium-Persulfate (APS) (Fisher cat. # BP179-25). Store at room temperature in the desiccator.
33. TEMED (Bio-Rad cat. # 161-0800). Store at room temperature.
34. 1X Western Blot Running Buffer: 25 mM Tris, and 192 mM Glycine. Store at room temperature.
35. X Western Blot Transfer Buffer: 25 mM Tris, 192 mM Glycine, and 20% methanol. Store at room temperature.
36. 4X Western Blot Sample Buffer (100 mL): Tris-HCl/SDS, pH6.8 (20 mL ddH<sub>2</sub>O, 3.03 g Tris Base, and 0.2 g SDS), 8 g SDS, 20 mL Glycerol, 0.1 g Bromophenol Blue. QS with ddH<sub>2</sub>O to 100 mL. This is a nonreducing buffer. For reducing conditions, you need to add 6.2 g of dithiothreitol (DTT) per every 100 mL. Aliquots into 1 mL and store at  $-20^{\circ}\text{C}$ .

### 2.3. Media

Store media at room temperature and all plates (and LBG medium) at  $4^{\circ}\text{C}$ . Use media and plates within 2 weeks if they contain antibiotics.

1. Luria Broth Base Media "LB" (Sigma cat. # L-3522).
2. LBG LB Media + 20 mM glucose.
3. S.O.C. Media at room temperature (Life Technologies, cat. # 15544-018).

4. Minimal Medium Plates.
5. 2-YT Medium (Life Technologies, cat. # 22712-020).
6. 2X YT-AG 100 µg/mL ampicillin and 2% glucose.
7. 2X YT-AI 100 µg/ml ampicillin and 1 mM IPTG.
8. 2X YT-G 2% glucose.

## 2.4. Others

1. Cluster tubes (96 tubes in a microtiter format, Costar, Cambridge, MA, cat. # 4411).
2. Plate sealer (Costar cat. # 6524).
3. RNase-free pipets (USA American Scientific Plastics cat. # 1010-8810 ).
4. RNase-free user tubes (Ambion cat. # 12400).
5. Colony lift butterfly S&S nitrocellulose membrane pore size 0.45 µm, diameter 82 mm (Schleicher & Schuell cat. # 401149).
6. RNase Alert (Ambion cat. # 1960).
7. Tranblot Tranfer Medium 0.2 µm Pure Nitrocellulose Membrane (Bio-Rad cat. # 162-0146).
8. Blot absorbent filter paper. (Bio-Rad cat. # 170-3932).

## 3. Methods

### 3.1 mRNA Isolation and Precipitation

The success of antibody cloning depends on the purity of the mRNA. The source of the mRNA can be isolated from either mouse antibody produced hybridoma, established cell lines or spleen-derived B lymphocytes. Any kit that will provide high-quality mRNA is recommendable. In our laboratory, we recommend the use of the Promega PolyATtract System 1000 because it isolates messenger RNA directly from crude cell or tissue lysates and eliminate the need for total RNA Isolation.

#### 3.1.1. mRNA Isolation from Hybridoma Cell Line ( $1 \times 10^7$ – $1 \times 10^8$ )

Before starting this protocol make sure that rotors and centrifuges are at room temperature to avoid precipitation of salts and detergents from solutions.

1. Remove the GTC extraction buffer, Biotinylated Oligo (dT) Probe, Nuclease-free Water, and SSC 0.5X Solution from the refrigerator and warm to room temperature. Preheat the dilution buffer to 70°C.
2. In a 50-mL sterile screw cap conical tube, add 41 µL of β-Mercaptoethanol (48.7%) per milliliter of extraction buffer and named "Extraction/BME Buffer." The final concentration of β-Mercaptoethanol is 2%. Use RNase-free pipet and wear gloves to reduce the chance of contamination.
3. Collect ( $1 \times 10^7$ –  $1 \times 10^8$ ) cells in a sterile 50 mL conical tube by centrifugation at 300g for 5 min. Wash the cell pellet with 23 mL of ice cold, sterile 1X phosphatase buffer saline (PBS) and centrifuge as above to collect the cells. Pour off the supernatant.



4. Add the "Extraction/BME Buffer" to the cells and mix by inversion the 50 mL tube 4 times. Homogenizing the cells by high speed for 15–30 s using a small homogenizer as Promega protocol recommends is optional.
5. Aliquot the preheated dilution buffer to a sterile tube and add 20.5  $\mu$ L of  $\beta$ -Mercaptoethanol (48.7%) per milliliter of dilution buffer. The final concentration of  $\beta$ -Mercaptoethanol is 1%. Add this to the homogenate and mix thoroughly by inversion. Add the biotinylated oligo (dT) Probe and mix well. Incubate this mixture at 70°C for 5 min.
6. Transfer the lysate to a clean, sterile 15 mL tube. Centrifuge at 12,000g for 10 min at room temperature to clean the homogenate of cell debris and precipitated proteins.
7. During the centrifugation, resuspend the SA-PMPs by gently rocking the bottle. The particles should appear as a homogeneous mixture and be fully suspended in the liquid. Transfer the 6 mL of SA-PMPs, to a sterile 50 mL conical screw cap tube away from the magnetic stand. Place the tube and the SA-PMPs on the magnetic stand. Slowly move the stand toward the horizontal position until the particles are collected at the tube side. Carefully pour off the storage buffer by tilting the tube so that the solution runs over the captured particles. Pouring in this manner decreases the chance of mixing the SA-PMPs into the solution again, which would decrease yields.
8. Resuspend the SA-PMPs to the original volume used (6 mL), in SSC 0.5X solution. Capture using the magnetic stand. Pour off the SSC solution as described in **step 7**. Repeat the washing a total of three times. Resuspend in the original volume (6 mL) with SSC 0.5X solution. Do not centrifuge these particles.
9. When the centrifugation of the homogenate is complete, carefully remove the supernatant with a sterile pipet, avoiding the pellet. The homogenate will be translucent.
10. Add the clear homogenate to the tube containing the washed SA-PMPs in SSC 0.5X solution and mix by inversion. It is important to add the homogenate away from the magnetic separation stand to ensure proper mixing.
11. Incubate the homogenate/SA-PMPs mixture at room temperature for 2 min. Capture the SA-PMPs moving the magnetic stand toward the horizontal position until the homogenate clears and then carefully pour off the supernatant as in **step 7**. Save the supernatant in a sterile tube on ice until certain that satisfactory binding and elution of the mRNA has occurred.
12. Resuspend the particles with 2 mL of SSC 0.5X solution by gently flicking the tube. Transfer the particle mixture to one of the 2 mL RNase-free user tubes. Capture the SA-PMPs by placing the tube in the magnetic stand. Carefully pipet off the SSC solution. Repeat this washing step twice. After the final wash, remove as much of the SSC solution as possible without disturbing the SA-PMPs cake.
13. To elute the mRNA, add 1 mL of RNase-free water and gently resuspend the particles by flicking the tube.
14. Magnetically capture the SA-PMPs by moving the magnetic stand toward the horizontal position, as before. Transfer the liquid containing the elute mRNA to a sterile RNase-free microcentrifuge tube.

15. To precipitate add 0.1 vol of 3M sodium acetate-DEPC treated and 1.0 vol of isopropanol to the eluate and incubate at  $-70^{\circ}\text{C}$  overnight.
16. Centrifuge at  $4^{\circ}\text{C}$  at (12,000g for 30 min. Resuspend the RNA pellet in 1 mL of 70% ethanol fresh made with RNase-free water and centrifuge again. Be careful when decanting the supernatant to avoid losing the RNA pellet.
17. For short-term storage (>30 days) let the pellet dry at room temperature for about 20 min and resuspend in 50  $\mu\text{L}$  of RNase-Free water and store at  $-70^{\circ}\text{C}$ . For long-term storage (>30 days), resuspend the mRNA pellet in 70% ethanol at  $-70^{\circ}\text{C}$ .
18. The concentration and the purity of the eluted mRNA can be determined by spectrophotometry. Determine the absorbance readings at 230, 260 and 280 nm ( $A_{230}$ ,  $A_{260}$ , and  $A_{280}$ ). Absorbance readings should be greater than 0.1 to ensure significance. To estimate the mRNA concentration, assume that a 40  $\mu\text{g/mL}$  mRNA solution will have an absorbance of 1 at 260 nm. Also, determine the  $A_{260}/A_{230}$  ratio which will provide information on the purity of the sample. An  $A_{260}/A_{230}$  ratio less than 2 indicates that GTC or  $\beta$ -Mercaptoethanol from the extraction buffer is still present. If this is the case, precipitate the RNA again.

### 3.2. First-Strand cDNA Synthesis

This First-strand cDNA synthesis is catalyzed by Moloney Murine Leukemia Virus reverse transcriptase. The use of random hexamers eliminates the need for immunoglobulin-specific primers or oligo (dT) primers. Using this random hexamers, the resulting cDNAs are of sufficient length (7 kbases or more) to clone the V regions from both the heavy and the light chain genes. We used the First-Strand cDNA Synthesis Kit from Pharmacia Biotech.

1. Place the 20  $\mu\text{L}$  mRNA sample.
2. Heat the mRNA solution to  $65^{\circ}\text{C}$  for 10 min, then chill on ice. Start the first-strand cDNA reaction within 2 min after placing on ice.
3. For each sample, label the tube as light chain or heavy chain in a 1.5 mL RNase-free microcentrifuge tube:

#### 3.2.1. Reverse Transcriptase Reaction

mRNA	20 $\mu\text{L}$
Primed First-Strand Mix	11 $\mu\text{L}$
DTT Solution	1 $\mu\text{L}$
RNase-free Water	X $\mu\text{L}$
	<hr/> 33 $\mu\text{L}$

1. Incubate for 1 h at  $37^{\circ}\text{C}$ . The completed First-strand cDNA reaction product is now ready for immediate PCR amplification.

### 3.3. Primary PCR Amplification

Alignment of known gene sequence from the variable domains have demonstrated that there are regions of conservation within the variable domain, particularly at the 5' and 3' termini. This has led to the determination of species-specific consensus sequences, which have been used in the design of PCR primers. In 1991, Clackson et al. described primers for the PCR amplification of mouse variable regions. However, the light chain from some monoclonal antibodies are difficult to amplify and they have since been redesigned as part of the Recombinant Phage Antibody System (RPAS) from Pharmacia, Biotech, and Mouse Ig-Prime System from Novagen. In our laboratories, we use the RPAS, and when the light chain can not be amplified, we use the Mouse Ig-Prime Kit that contains primers for the  $\mu$ , all  $\gamma$ ,  $\kappa$ , and  $\lambda$  light chain. The First-strand antibody cDNA is used as a template for PCR amplification to generate suitable quantities of heavy (~340 bp) and light (~325 bp) chain DNA cloning.

1. Add to 500  $\mu$ L microcentrifuge tubes label as "light chain" or "heavy chain" the following reagents.

#### 3.3.1. Light Chain PCR

First-strand Reaction	33 $\mu$ L
Light Primer Mix	2 $\mu$ L
Sterile distilled water	64 $\mu$ L
	<u>99 <math>\mu</math>L</u>

#### 3.3.2. Heavy Chain PCR

First-strand Reaction	33 $\mu$ L
Heavy Primer 1	2 $\mu$ L
Heavy Primer 2	2 $\mu$ L
Sterile distilled water	64 $\mu$ L
	<u>99 <math>\mu</math>L</u>

1. Mix with a micropipettor and spin briefly.
2. Overlay each reaction with two drops of mineral oil. Place the tube in a thermocycler and heat at 95°C for 5 min.
3. Add 1  $\mu$ L of AmpliTaq DNA polymerase to each reaction beneath the mineral oil, using separate pipets tips for each addition.
4. Run 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min.

### 3.4. Purification of Primary PCR Products

Before performing the assembly reaction, it is very important that the heavy and the light-chain PCR products are isolated from the other reaction components. Purification of the DNA from an agarose gel can be done. In this context, our laboratory used the QIAquick Gel Extraction Kit from Qiagen. This QIAquick system combines the convenience of microspin technology with the selective binding properties of a specially adapted silica-gel membrane.

#### 3.4.1. Agarose Gel Electrophoresis

1. Prepare a 1% agarose gel in 1X TAE Buffer with wells sufficient to accommodate 100  $\mu$ L samples.
2. Remove ~90  $\mu$ L from the PCR amplification reaction tube and transfer to a new centrifuge tube.
3. Save as a backup 20  $\mu$ L of the PCR product of each amplification at  $-20^{\circ}\text{C}$ . Add 11  $\mu$ L of 6X Loading Dye (see Note 4.1., item 7) to the remanding 70  $\mu$ L of PCR amplification reaction.
4. Load the PCR amplification reaction mix with the loading buffer in the well. Electrophoreses at 80 V until the bromophenol blue dye has migrated  $\sim 1/3$  the length of the gel.

#### 3.4.2. QIAquick Gel Extraction

1. Excise the DNA fragment (heavy chain ~340 bp and light chain ~325 bp) from the 1% agarose gel with a clean scalpel under a long wavelength ultraviolet (UV) light in the transilluminator. Try to minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a tube. Add 3 vol of Buffer QG to 1 vol of gel.
3. Incubate at  $50^{\circ}\text{C}$  for 10 min. To help to dissolve the gel you can mix by flicking or use a thermomixer. Is very important to solubilize the agarose gel completely. The maximum amount of agarose per column is 400 mg. At this time it is very important, to check the pH because the adsorption of the DNA to silica depends on the pH. Binding efficiency during the adsorption step is typically 95% if the pH is  $<7.5$ , and is reduced drastically at higher pH. However if the loading mixture pH is  $>7.5$ , it can be lowered by adding 10  $\mu$ L of 3M sodium acetate pH 5.0.
4. Place a QIAquick spin column in a 2 mL collection tube and load the sample.
5. Centrifuge for 1 min at 6000g ( $\sim 9000$  rpm) in a eppendorf centrifuge. The maximum loading volume of the column is 800  $\mu$ L. For larger sample volumes, multiple loading of the column are necessary.
6. Discard flow-through and place the QIAquick column back in the same collection tube.
7. Add 0.5ml of Buffer QG to the QIAquick column and centrifuge for 1 min.
8. Centrifuge for 1 min at 6000g ( $\sim 9000$  rpm) in a eppendorf centrifuge.
9. To wash, add 0.75 mL of Buffer PE to the QIAquick column and let the column stand 5 min.

10. Centrifuge for 1 min at 6000g (~9000 rpm) in a eppendorf centrifuge.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 10,000g (~13,000 rpm).
12. Place the QIAquick column to air dry for 15–20 min.
13. To elute, add 50  $\mu$ L of sterile water to the center of the column and let column stand for 5 min. DNA is an acid and will undergo autocatalytic degradation in the absence of a buffering agent and must therefore be stored at  $-20^{\circ}\text{C}$  when eluted with water.
14. Centrifuge the QIAquick column for 1 min at 10,000g (~13,000 rpm).

### **3.5. Gel Quantification of Purified Heavy and Light-Chain From PCR Amplification Products**

The success of assembly and fill-in reactions is dependent upon the molar concentration of both heavy- and light-chain PCR products. Agarose gel electrophoresis of aliquots of the purified heavy- and light-chain products alongside a known amount of  $V_H$  Marker (provided by the RPAS Kit) gives a visual estimate of the relative amounts of the fragments based on their band intensity in an ethidium-bromide-stained gel.

1. Prepare a small 1.5% TEA Agarose gel with 3 mm wells, and add ethidium bromide at the final concentration of 0.5  $\mu\text{g/mL}$ .
2. To prepare marker, add 2.5 or 5  $\mu\text{L}$  (12.5 or 25 ng) of  $V_H$  marker (provided by the RAPS kit) with 1  $\mu\text{L}$  of loading dye.
3. Load in separate wells 10  $\mu\text{L}$  of each heavy- and light-chain with 2  $\mu\text{L}$  of loading dye.
4. Load in separate well 1  $\mu\text{g}$  of 100 bp ladder.
5. Electrophoreses TEA Agarose gel at 80 V for 30 min.
6. Photograph the gel under UV light. It may be necessary to overexpose the picture to visualize the marker and the heavy and light chain.
7. Compare the intensity of the heavy- and light-chain products with the  $V_H$  Marker.
8. Estimate the volume of purified heavy-chain product that corresponds to 50 ng. Estimate the volume of purified light-chain product that corresponds to 50 ng. This will be your 1:1 ratio.

### **3.6. Assembly and Fill in Reactions**

In the assembly reaction, the heavy and light chain of the antibody are joined into a single-chain antibody with a linker DNA ([Gly<sub>4</sub>Ser]<sub>3</sub>). When the linkers anneal to the heavy- and light-chain DNA, they prime a fill-in reaction in the presence of AmpliTaq DNA polymerase. For this reaction to proceed efficiently, approximately 1:1, 1:2, 1:3, 1:4, and 1:5 ratios of heavy to light chain DNA must be added. If you do not have enough PCR products, you can reamplify heavy and light chain.

1. Add the following components to a 500  $\mu$ L microcentrifuge tube:

Heavy Chain Product (50 ng)	X $\mu$ L
Light Chain Product (50 ng, 100 ng, etc.)	X $\mu$ L
Linker-Primer Mix	4 $\mu$ L
10X PCR Buffer I (without $MgCl_2$ )	5 $\mu$ L
dNTP Mix (10 mM of each)	5 $\mu$ L
25 mM $MgCl_2$	5 $\mu$ L
AmpliTaq DNA Polymerase (5 U)	1 $\mu$ L
Sterile distilled water	to 50 $\mu$ L
	50 $\mu$ L

2. Mix with a micropipettor and spin briefly.
3. Overlay each reaction with two drops of mineral oil. Place the tube in a thermocycler and heat at 95°C for 5 min.
4. Add 1  $\mu$ L of AmpliTaq DNA polymerase to each reaction beneath the mineral oil, using separate pipette tips for each addition.
5. Run 7 cycles of 94°C for 1 min, 63°C for 4 min. 1 cycle of 72°C for 10 min.

### 3.7. Second PCR Amplification and Purification

It is necessary to amplify the assembled scFv DNA for further cloning steps. In this second PCR, amplification restriction sites are added. These restriction sites are used to clone into the phagemid vector. The restriction site primers (RS primers) contain the *Sfi* I annealing with the heavy chain in the 5' end, and the *Not* I site with the light chain in the 3' end.

1. Add to the Assembly and fill-in reaction the following:

AmpliTaq (5 U)	1 $\mu$ L
10X Buffer II (with $MgCl_2$ )	5 $\mu$ L
dNTP Mix (10 mM)	2 $\mu$ L
RS Primers Mix	4 $\mu$ L
Sterile distilled water	39 $\mu$ L
	50 $\mu$ L

2. Mix with a micropipettor and spin briefly.
3. Overlay each reaction with two drops of mineral oil. Place the tube in a thermocycler and heat at 95°C for 5 min.
4. Run 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. 1 cycle of 72°C for 10 min.
5. Run a 1.5% TEA Agarose gel at 80 V for 30 min. A predominant band ~750 bp in size should be present. Some heavy and light chain monomers may be visible.
6. Follow the QIAquick gel extraction kit to extract the scFv DNA from the gel, and follow it by gel quantification.

### 3.8. Restriction Digestion and Purification

The assembly scFv contains the *Sfi* I and *Not* I sites introduced by the RS primer in the second PCR amplification. The scFv DNA has to be digested in order to be cloned into the phagemid plasmid pCANTAB 5E.

#### 3.8.1. *Sfi* I Digestion

1. Gel purified scFv product (0.25-1 µg) up to 70.0 µL  
10X *Sfi* I Restriction Buffer 8.5 µL  
*Sfi* I (20 U per reaction) X µL  
Sterile distilled water to 85.0 µL  
85.0 µL
2. Mix gently and overlay with two drops of mineral oil. Incubate overnight at 50°C.
3. The next day equilibrate the *Sfi* I digested sample to room temperature and spin briefly.

#### 3.8.2. *Not* I Digestion

1. Add to the scFv DNA *Sfi* I digested sample the following:  
10X *Not* I Restriction Buffer 12.7 µL  
*Not* I (20 U per reaction) X µL  
Sterile distilled water to 85.0 µL  
85.0 µL
2. Mix gently and overlay with two drops of mineral oil. Incubate during the day (8 h to overnight) at 37°C.
3. Follow the QIAquick gel extraction kit to extract the scFv DNA from the gel, and follow it by gel quantification.

### 3.9. Ligation of the scFv cDNA into the Phagemid Plasmid pCANTAB5E

McCafferty et al. described in 1990 the fd-CAT1 original phage vector for antibody display (27). These vectors contain all the genetic information encoding the phage life cycle. In this context, an alternative system has been used up to now. This system involves cloning into phagemid vectors that contain a copy of the gene 3 and phage packaging signal sequence. Thus, antibody fragments can be displayed as a fusion with the gene 3 protein and the genetic information is packaged thanks to the packaging signal. In our laboratory we have used the phagemid vector pCANTAB 5 E included in the Pharmacia RPAS kit. This vector allows cloning of antibody genes into *Sfi* I and *Not* I sites. This vector incorporates an amber codon between the C-terminus of the

cloned scFv and the start of gene 3 sequence allowing the recombinant antibody to be made as a soluble protein. This vector also includes a peptide tag, allowing the detection of the single-chain antibody.

1. The assembled product should be gel-quantitated as described in **Subheading 3.5**.
2. For ligation of the scFv gene to the pCANTAB 5 E vector (provided by the RPAS Kit from Pharmacia), add the following into a 1.5 mL microcentrifuge tube:
 

scFv gene Fragment (100 ng)	X $\mu$ L
pCANTAB 5 E vector (250 ng)	5.0 $\mu$ L
0X Ligation Buffer	1.5 $\mu$ L
T4 DNA Ligase (5 U)	1.0 $\mu$ L
Sterile distilled water	to 15.0 $\mu$ L
	<hr style="width: 100px; margin: 0;"/> 15 $\mu$ L
3. Incubate all reactions at 14°C overnight.
4. Next day, add 2  $\mu$ L of sodium acetate and 500  $\mu$ L of 95% ethanol.
5. Incubate at -70°C 4 h.
6. Spin at 14,000 rpm for 30 min at 4°C.
7. Discard supernatant carefully not to loss the pellet and wash with 500  $\mu$ L of 70% ethanol.
8. Spin at 14,000 rpm for 20 min at 4°C.
9. Discard supernatant carefully and let the pellet air dry.
10. Resuspend in 10  $\mu$ L of sterile water (for electroporation you need a low ionic strength).

### 3.10. Transformation

Two *E. coli* strains are used for preparation of single chain antibody libraries. To produce phage-displayed recombinant antibodies, competent TG1 cells are used. This host strain produces a suppressor tRNA which allows readthrough (suppression) of the amber stop codon. The switch to soluble recombinant antibody production is accomplished by using the *E. coli* strain HB2151 cells. The HB2151 cells are the nonsuppressor strain, allowing the recognition of the amber stop codon; thus, only soluble single chain antibodies can be produced. The *E. coli* TG1 and HB2151 are supplied as lyophilized cultures in the Expression Module/RPAS Kit from Pharmacia for phage rescue. These *E. coli* cells will need to be prepared for an electroporation protocol. We recommend that 1 ng of uncut supercoiled vector (pcDNA3 or other irrelevant vector) be used to determinate the efficiency of the competent cells.

#### 3.10.1. Preparation of HB2151 for Electro-Transformation

1. Inoculate 1 L of broth with a 1/100 volume of a fresh overnight culture.
2. Grow cells at 37°C in a shaking incubator until the  $OD_{600}$  = 0.5–1.0.
3. Chill the cells on ice for 15–30 min.



4. Transfer cell culture to sterile centrifuge tubes and centrifuge in a cold rotor at 4000g (max) for 15 min.
5. Remove supernatant and discard. Resuspend cell pellet in 1 L of cold sterile 10% glycerol.
6. Centrifuge again as in **step 4**.
7. Remove supernatant and resuspend cell pellet in 500 mL of cold sterile 10% glycerol.
8. Centrifuge as in **step 4**.
9. Remove supernatant and resuspend cell pellet in 250 mL of cold sterile 10% glycerol.
10. Centrifuge as in **step 4**.
11. Resuspend cells in a final volume of 2 to 3 mL of cold 10% glycerol and dispense 40  $\mu$ L per sterile eppendorf tube.
12. Quick freeze cells in dry ice/ethanol bath before storing at  $-70^{\circ}\text{C}$ . These cells should be good for six months.

### 3.10.2. Electroporation

1. Thaw the HB2151 cells ( $-70^{\circ}\text{C}$ ) on ice.
2. Add 1 to 2  $\mu$ L of DNA (solution of DNA should be low in ionic strength) ligation mix and let sit on ice about 1 min.
3. Set the electroporator at 1.8 kV.
4. Transfer the cell mixture to prechilled cuvettes (make sure the suspension is at the bottom of the cuvet).
5. Charge the pulser and then discharge (this should produce a pulse with a time constant of between 4 and 5 ms).
6. Add about 500  $\mu$ L of S.O.C. media to cuvet, then transfer contents of cuvet to a snap capped tube containing 500  $\mu$ L of media.
7. Incubate tube at  $37^{\circ}\text{C}$  (shaking) for one h.
8. Plate onto 2xYT-AG selective media.
9. Incubate overnight at  $30^{\circ}\text{C}$ . These colonies will be used for the modified colony lift assay. Make sure that these colonies are well isolated: ~200–300 colonies per plate.

### 3.11. Modified Colony Lift Assay

The following protocol describes a colony lift assay whereby scFv-expressing clones of *E. coli* can be rapidly identified. Because of the large number of colonies that can be simultaneously screened, it may be possible to recover not a single positive clone. Thus, we recommend that this step be repeated at least twice with each positive colon. After each panning, we strongly recommend a PCR amplification of each positive clone with the RS primer as indicated in **Subheading 3.7**. Make sure you make a glycerol stock as soon as possible of each positive clone (28).

### 3.11.1. Antigen-Coated Membrane

1. Dilute 50 µg of the target protein in 1 mL of PBS.
2. Coat S&S nitrocellulose membrane adding the 1 mL of protein in the middle of the membrane and gently move it around until the entire membrane is wet. This side of the membrane is where your antigen is, make sure that you always have it face up.
3. Block filter with 5% milk in PBS for one h.
4. Rinse the membrane with PBS and let it dry for 15 min.
5. Lay the antigen-coated membrane on top of the 2xYT-AI agar plate.

### 3.11.2. Master Membrane

1. Put a new nitrocellulose membrane on top of the plate that has your colonies in the 2xYT-AG. Note: The colonies have to be well isolated. Invert "colony membrane" (colony-side up), and place on top of your "antigen-coated membrane" in the 2x-YT-AI.
2. Incubate at 30°C overnight.
3. Lift colony filter and place colony-side up onto a fresh 2x-YT-AG plate at 4°C until you have develop the antigen-coated membrane and be ready to select.

### 3.11.3. Development of Antigen-Coated Membrane

1. Place antigen-coated membrane (face-up) in a Petri dish and wash 5 min three times with PBS-0.1% Tween20.
2. Block membrane in 5% nonfat milk in PBS for 1 h.
3. Wash membrane with PBS-0.1% Tween20 10 min three times.
4. Add anti-E Tag-HRP antibody 1 : 1000 in 5% nonfat milk 1 h at room temperature.
5. Remove the antibody and wash 15 min three times with PBS-0.1% Tween20.
6. Develop with 4CN substrate (Sigma cat. # C-6788).

4CN substrate: Dissolve one tablet of 4-Choloro-1-Naphthol (4C1N) (30 mg) in 10 mL of methanol. This reagent is good for 2 wk when kept in the dark at 4°C. Immediately prior to development add 2 mL of stock 4CN/MeOH to 10 mL of triethanolamine saline pH 7.5. Add 5 µL of 30% H<sub>2</sub>O<sub>2</sub>. Neutralize with water (rinse).

### 3.12. Production of Soluble Antibodies (Periplasmic Extract)

The single chain antibody is clone into to the pCANTAB 5 E phagemid vector and it can be expressed as a soluble protein from this vector. Although the localization and concentration of the scFv will vary, most of the cases the bacterial periplasmic extract will yield the highest concentration of functional scFv antibodies (28).

1. Grow up each of the positive clones in 2 mL of 2x YT-AG at 30°C to log phase (OD<sub>550</sub> = 0.3–0.4).

2. At this point make a glycerol stock, and a PCR as indicated in **Subheading 3.7.** to check the presence of the scFv.
3. Spin down 1500g for 5 min.
4. Resuspend cells in 2 mL of 2x YT-AI at 30°C overnight.
5. Spin 1500g 20 min in a clinical centrifuge and aspirate media.
6. Resuspend in 400 mL of cold 1 mM EDTA in PBS.
7. Incubate at 4°C for 30 min with regular movement.
8. Spin at 16,000g for 10 min.
9. Transfer supernatant (soluble scFv) to a new tube.

### **3.13. Detection and Binding Specificity of E-Tagged scFvs**

Enzyme-linked immunosorbent assays (ELISA) can be used to characterize the positive clones obtained from the colony lift assay. In this ELISA procedure, HRP/Anti-E Tag conjugate is used to detect E-tagged scFv from the periplasmic extract bound to the antigen-coated microtiter well. Because the ELISA is quantitative in nature, the signal will vary with the expression level and affinity of the scFv. When the positive clones are identified these single chain antibody can be cloned into eukaryotic expression vector for intracellular expression.

#### **3.13.1. Coating**

1. Coat wells of the microtiter plate for ELISA with 200 µL of target antigen in PBS (pH 8.0–8.5) yielding 100 ng to 10 µg per well.
2. Coat wells with appropriate controls (irrelevant protein).
3. Incubate overnight at 4°C, covered.
4. Shake out contents of plate and rinse once with PBS using a squirt bottle.

#### **3.13.2. Blocking**

1. Add 200 µL of 3% BSA and 1% gelatin in PBS to each well.
2. Incubate 1–2 h at room temperature.
3. Shake out contents of plate and rinse once with PBS using a squirt bottle.

#### **3.13.3. scFv Periplasmic Extract**

1. Add 100 µL of periplasmic extract to each well.
2. Incubate at room temperature for 2 h or at 4°C overnight.
3. Wash three times for 15 min with PBS.

#### **3.13.4. Conjugated Antibody**

1. Dilute HRP/Anti-E Tag Conjugated 1:1000 in 2% BSA, and add 100 µL to each well.
2. Incubate at room temperature for 1 h.
3. Wash three times for 15 min with PBS.

### 3.13.5. Developer

1. Develop with 1X ABTS substrate. Add 100  $\mu$ L developer to all wells and allow color to develop 15–30 min or until color (green) reaction has occurred.
2. The reaction can be read in a microtiter plate reader set at 405–415 nm. If a microtiter plate reader is not available, a spectrophotometer set at an absorbency of 410 nm can be used to quantitate the results. The absorbency reading for your antigen should be at least two to three times higher than the absorbency reading for the negative control.

### 3.14. Western Blot Analysis of E-Tagged scFvs

The principle behind SDS-PAGE is the electrophoretic separation of proteins based on mobility in an electrical field as well as their molecular size. Bigger proteins move through the gel slower than smaller ones. You can vary the concentration of the acrylamide to separate proteins of low, midrange or high-molecular size. For instance, if you are interested in a 29-kDa protein, use a 12–15% gel.

#### 3.14.1. Electrophoresis

1. Set up the plates by placing the spacers on either side and clamping the white side-clamps by turning the screws tightly.
2. Install the plates into the bottom mold stand and turn the screws to clamp it down into the base.
3. Fill up with water to test seal and to insure that no leaks occur.
4. Pour the separating gel and let harden 30 min to 1 h. Place a layer of saturated isopropanol over the gel.

Resolving gel: 0.375M Tris, pH8.8 (~30 min)

		7%	8%	9%	10%	12%	15%	20%
1.5M Tris (pH8.8)	mL	2.5	2.5	2.5	2.5	2.5	2.5	2.5
40% Acrylamide	mL	1.75	2.0	2.25	2.5	3.0	3.75	5.0
ddH <sub>2</sub> O	mL	5.6	5.35	5.1	4.85	4.35	3.6	2.35
10% SDS	100 $\mu$ L							
10%APS (make fresh)	50 $\mu$ L							
TEMED	2.5 $\mu$ L	1.5 $\mu$ L						

5. Empty out the saturated isopropanol by inverting the plates and then pour in the stacker gel. You can either load the stacker gel into the plates using a 5 mL pipet with the combs in place or pour the stacker gel in and then place the comb between the glass plates. Be sure there is no bubbles in the bottom of the comb.

Stacker 4%: 0.125M Tris, pH 6.8 (~25 min)

0.5M Tris (pH6.8)	1.0 $\mu$ L
40% Acrylamide	0.4 $\mu$ L

ddH <sub>2</sub> O	2.54 $\mu$ L
10% SDS	40 $\mu$ L
10% APS	20 $\mu$ L
TEMED	4 $\mu$ L

6. Allow to set 30–60 min.
7. Gently pull out comb.
8. Place the top chamber over the gels and remove the bottom screws. Now put these screws into the top chamber and turn to tighten.
9. Fill up top chamber with 500 mL of 1X Western Blot Running Buffer.
10. Load samples using round sequencing pipet tips and put lid on. Prior to loading, samples should be boiled in 1X reducing Western Blot Sample Buffer.
11. Run the gels at 150 V for 1 h.

### 3.14.2. Western Transfer

Here the principle is to transfer proteins to a solid support for Western blotting. The entire assembly is done between the paddles of the transfer apparatus. Place the paddle with the protruding nubbin down first, next the backing, then all the things below followed by more backing and then the other paddle without the nubbin.

1. Carefully remove the gel from the plates.
2. Wet two pieces of blot absorbent filter paper, 1 nitrocellulose membrane, and the sponges in 1X Western Blot Transfer Buffer.
3. Lay the gel on top of the sponge and blot absorbent filter paper.
4. On top of gel, place a pre-wet in 1X Western Blot Transfer Buffer piece of nitrocellulose membrane and one blot absorbent filter paper.
5. Place one more piece of pre-wet blot absorbent filter paper on top of the nitrocellulose, and then one sponge.
6. Snap the paddles together.
7. Load into the transfer chamber which has been filled with 1 L of 1X Western Blot Transfer Buffer.
8. Snap on power pack lid which has the back facing towards you (need to transfer from negative to positive). Make sure that the two protruding male electrodes in the bottom transfer tank are secured into the powerpack lid.
9. Run the transfer at 100 V for 1 h.

### 3.14.3. Developing Western

1. Block with PBS with 5% nonfat milk and 1% BSA for 30 min to overnight.
2. Add your primary antibody (HRP/Anti-E Tag) at 1 : 3000 in PBS 5% nonfat milk and 1% BSA at 4°C shaking overnight.
3. Wash with PBS for 30 min to 1 h, changing the PBS every 10 min (you will never overwash, but you can under-wash).
4. Develop with 4CN substrate as indicated in **Subheading 3.11**.

### **3.15. Intracellular Expression of the scFv**

It has recently been shown that scFv molecules may be expressed intracellularly in eukaryotic cells by gene transfer of scFv cDNAs. The encoded scFv may be expressed in the target cell and localized to specific, targeted subcellular compartments by appropriate signal molecules. Invitrogen has mammalian expression vectors that allow targeted of your protein to different subcellular compartment. Each of the vectors in the pShooter collection incorporates a signal sequence that will direct the scFv to a specific subcellular location. These eukaryotic expression vectors contain a strong mammalian CMV promoter, and a C-terminal tag for rapid detection.

#### **3.15.1. cDNA scFv Cloning in Eukaryotic Expression Vector**

Excised the scFv cDNA from the pCANTAB 5 E phage vector utilizing the *Sfi I* and *Not I* restriction sites as indicated in **Subheading 3.8**.

Set up ligation reactions as indicated in **Subheading 3.9**., follow up by transformation into *E. coli* competent cells of your choice (DH5 $\alpha$ , SURE) as described in **Subheading 3.10**.

## **4. Notes**

We have identified 5 major sources of problems throughout the recombinant single chain antibody system: 1) quality of isolated mRNA; 2) PCR amplification of  $V_H$  and  $V_L$ ; 3) assembly reaction of the scFv; 4) ligation of the scFv to the phegamid plasmid pCANTAB5E; 5) Modified Colony Lift Assay. In this section, we will give a guide and how to identify this problems and troubleshoot them.

### **4.1. Quality of Isolated mRNA**

The success of antibody cloning depends on the quality (purity) of the mRNA. Highly pure mRNA is required as starting material for generating a recombinant antibody. In our experience, the PolyATtract System 1000 isolates this high-quality mRNA. This system from Promega utilized Promega's MagneSphere technology for the purification of poly (A)+RNA, eliminating the need for oligo (dT) cellulose columns. The successful isolation of intact mRNA four important steps must be performed: 1) effective disruption of cells or tissue, 2) denaturation of nucleoprotein complex, 3) inactivation of endogenous ribonuclease (RNase) activity, and 4) purification of RNA away from contaminating DNA and proteins. The most important of this is the inactivation of RNases. This system combines the disruptive and protective properties to inactivate ribonuclease present in the cell extract, as well as high stringency method to obtain pure mRNA. The isolation of this high-quality mRNA with

this kit is suitable for all molecular biology applications and the yields of poly (A)+RNA is two-fold greater than with other methods.

Special precautions must be observed to avoid degradation of mRNA by RNases.

1. Your hands are a major source of RNases so always wear gloves when working with RNA.
2. The second major of RNase contamination is bacteria or molds that may be present on airborne dust particles, thus glassware, etc., that is to be used for RNA preparations should be purchased new and only used for working with RNA.
3. Used RNase Zap (Ambion) to spray all work surfaces and equipment.
4. All glassware should be washed thoroughly and soaked in a 0.1% solution of diethyl pyrocarbonate (DEPC) (inhibits RNases) for at least 15 min, autoclaved, and then baked in an oven at 250°C for 3 h (to destroy traces of DEPC—DEPC decomposes into CO<sub>2</sub> and ethanol when heated).
5. Whenever possible, use sterile single-use plastic ware instead of glassware.
6. Chemicals should be reserved for working with RNA and always handled with DEPC treated spatulas.
7. All solutions should be treated with DEPC prior to use. The solutions should be adjusted to 0.1% in DEPC, shaken or stirred for at least 15 min and then autoclaved. Because DEPC decomposes in the presence of Tris, all solutions containing Tris should *not* be DEPC-treated but instead be made up with DEPC-treated and autoclaved ddH<sub>2</sub>O. Likewise solutions that are not to be autoclaved should always be made up with DEPC-treated water or solutions. You may want to check with RNase Alert from Ambion your solution before using them.

#### 4.2. PCR Amplification of V<sub>H</sub> and V<sub>L</sub>

It can be possible that following the primary PCR amplification (**Subheading 3.3.**) step you can not see the light- and heavy-chain products. Since we have used the PolyATtract System 1000 we have not had this problem, however if you are using other RNA purification methods the reason to fail this first PCR amplification can be as follows:

1. The purity of the mRNA is not adequate: this problem may be resolved by using an RNA extraction kit followed by mRNA purification kit.
2. Ribonucleases are present in the mRNA preparation: follow RNase-free precautions.
3. The hybridoma was no longer producing antibody: check the hybridoma for the presence of soluble antibody.
4. The primers are annealed nonspecifically to nucleic acids contaminating the mRNA: you will see a smearing in the gel electrophoresis, better purification of the mRNA preparation is required.
5. If you only can see the heavy chain but not the light chain it is because the Pharmacia mouse scFv module kit contains only the kappa light chain primers

since few lambda light chain sequence have been determined (~5%). The Novagen Mouse Ig-Primer Set (Novagen cat. # 69831-3) will allow you to PCR the light chain, however you may have to follow their protocols to assemble the scFv, and continue in **Subheading 3.9**.

Special precautions must be observed to avoid PCR contamination:

1. Use the PCR hood when making reagents for PCR or putting together reactions. The pipetmen in the hood have never been used to pipet DNA and should never be removed from the hood. Likewise, DNA—or anything that has been near DNA (dirty gloves, tube racks, etc.)—should never be brought into the PCR hood.
2. Always make up your PCR reagents with stocks and solutions that have never been out on the lab bench. Always use disposable tubes and pipets to make up stocks. It's often a good idea to make up a large batch of buffer or dNTPs, test them to make sure they're clean, and divide them into small aliquots.
3. If you suspect that your PCR reagents (buffer, dNTPs, or water) are contaminated, try irradiating them on the short wave UV box (about 3–5 min). This will crosslink any contaminating DNA. You can also UV zap some primers without affecting their ability to prime, but this is primer specific. Do not UV zap the enzyme.
4. As long as your reagents are clean, most contamination occurs when you add the template to your tubes. Take care in opening the tubes that contain your DNA template. A little bit of DNA aerosolized onto your glove can easily be transferred to your PCR tube. Use pipet tips with filters or positive displacement pipets to add your template-aerosolized DNA in pipetman is the major source of contamination.
5. Always include a negative control. Make sure to treat it like the other samples (e.g., add 1  $\mu$ L of water at the lab bench with the pipetman you are using to add DNA to the other tubes).
6. For RT for PCR you should include two negative controls. First, everything except RNA is added ( $H_2O$  control). (Be sure to actually add water to this tube at the same time you are adding RNA to the other tubes); and second, when RNA is added, but no RT is included (this ensures that there is no contamination of your RNA).

#### **4.3. Assembly Reaction of the scFv**

The assembly and fill in reaction of the scFv is with no doubt the trickiest step of all. We will give you some tips that with our experience have made this step easy.

1. Make sure a high quality of the cDNA of  $V_L$  and  $V_H$  is used as a start material. The QIAquick Gel Extraction Kit from Qiagen will give you this high-quality DNA. Two modifications we introduce to their protocol: 1) Skip the isopropanol step after the solubilization of the agarose gel. It is very difficult to completely



dry the isopropanol and it can interfere with downstream reactions and 2) let the column air dry at room temperature for 20 min before elution of the DNA, thus no ethanol will be present.

2. The Pharmacia protocol suggest for the assembly reaction of the scFv an equivalent amount of the heavy- and light-chain products, however, in our experience for this reaction to proceed efficiently we add an excess of light-chain product. We proposed that approximately 1:1, 1:2, 1:3, 1:4, and 1:5 ratios of heavy- to light-chain DNA must be used for this assembly reaction to be successful.
3. The final concentration of the dNTPs in the assembly reaction is critical in this step. We have modified the dNTPs molarity to 10 mM using 1/10 of the total volume in the assembly reaction as indicated in **Subheading 3.6**.

#### **4.4. Ligation of the scFv to the Phagemid Plasmid pCANTAB5E**

After ligation of the scFv in the phagemid vector pCANTAB 5 E we recommended that **Subheadings 3.9.** through **3.12.** be done within 48 hr. It is important that you make a glycerol stock of the positive clones as soon as possible. PCR amplification of the scFv library after this time, demonstrated different scFv cDNA sizes.

#### **4.5. Modified Colony Lift Assay**

Some antibodies produced by hybridoma cells cannot recognize their respective antigen if this is immobilized onto nitrocellulose. You need to try several times by colony lift assay if your hybridoma recognize the antigen-bound to the membrane. If this is the case, the colony lift assay cannot be used to detect the recombinant antibodies, and you may need to work with the Recombinant Phage Antibody System with the Phage Antibody Library by Pharmacia.

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Table 32-9 ■ SOME INBORN ERRORS OF PROTEINS THAT CIRCULATE IN BLOOD

FUNCTIONAL CLASS	PROTEIN	PHENOTYPE
Transport	Ceruloplasmin	Wilson's disease
	Albumin	Analbuminemia
	Hemoglobin	Hemoglobinopathies
	$\alpha$ -Lipoprotein	Analphalipoproteinemia
	$\beta$ -Lipoprotein	Abetalipoproteinemia
	Transcobalamin II	Megaloblastic anemia
Hormones	Growth hormone	Pituitary dwarfism
	Insulin	Diabetes mellitus (insulin-dependent)
	Somatomedin	Pituitary dwarfism
Coagulation	Factors I–XIII	Coagulopathies
	Kininogen	Kininogen deficiency
	Prekallikrein	Prekallikrein deficiency
Immune system	Complement components	Hypocomplementemias
	Immunoglobulins	Hypogammaglobulinemias
Inhibitors	$\alpha$ 1-Antitrypsin	Pulmonary emphysema and/or cirrhosis
	C'I esterase inhibitor	Angioneurotic edema

Abnormal structural proteins produce inborn errors such as Marfan syndrome (fibrillin), osteogenesis imperfecta (collagen type I), spondyloepiphyseal dysplasia (collagen type II), and Sack's syndrome (collagen type III) (see Chapters 215 to 218). These disorders exemplify class 4 of inborn errors of metabolism (see Table 32-1). The enzymes involved in post-translational processing of these proteins may also cause these syndromes. An example is Ehlers-Danlos syndrome type VI in which collagen lysylhydroxylase deficiency produces excess poorly hydroxylated collagen. Inborn errors of matrix proteins are exemplified by disorders of collagen metabolism. More than 20 different genes dispersed on 9 chromosomes are currently known to code for more than 11 different types of collagen. These disorders are detailed in Chapters 215 to 218, and 283.

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## 33 GENE THERAPY

Jesús Gómez-Navarro ■ Guadalupe Bilbao  
■ David T. Curiel

Gene therapy is a relatively new method of therapeutic intervention targeted at the level of cellular gene expression. In this approach, altering a pathophysiologic state is achieved by delivering nucleic acids into a cell. These nucleic acids may be genes, portions of genes, oligonucleotides, or ribonucleic acid. In conventional therapeutics, as in pharmacotherapy, altering a cell or tissue phenotype is accomplished by altering cell physiology or metabolism at the level of protein expression. For gene therapy, this is accomplished by changing the pattern of expression of the genes whose products may thus achieve the desired effect on the cellular phenotype. From a conceptual standpoint, gene therapy strategies may offer the potential to achieve a much higher level of specificity of action by virtue of the highly specific control and regulatory

mechanisms of gene expression that may be targeted in this technique. Additionally, interceding at an earlier stage in disease pathogenesis may offer greater potential to achieve fundamental changes in phenotypic parameters of disease with a more favorable outcome. Lastly, using the body to produce therapeutic proteins, potentially in only certain tissues, has practical advantages of its own.

Gene therapy was initially conceptualized as a method to treat acquired genetic diseases. In this regard, more than 5000 monogenic disorders exist in which the entirety of the disease state may be attributed to a single lesion at a specific genetic locus. Replacing or augmenting a defective gene by delivering its wild-type counterpart thus offers a potential means to rectify definitively the pathogenic basis of the disease state. Inherited genetic diseases, however, are not the only logical targets for gene therapy. The underlying basis for a variety of acquired disorders may be shown to be accumulated lesions in specific genetic loci, as in malignancies associated with mutations in dominant and recessive oncogenes. In these instances also, if the pathogenic basis is established to be lesions in cellular genes, a logical strategy is replacing or adding the mutated genes with the wild-type counterpart to perform the deficient function.

The indications for this form of therapy must be established. The first and foremost criterion for any gene therapy is that the aberrant gene being targeted must be well characterized. In addition, it must be shown that the defined genetic abnormalities are the basis of the observed pathogenesis of the disease state. If the logic of genetic intervention thus exists, clearly defined endpoints of the therapy intervention must exist and an alternate, effective therapy for the targeted disease must not exist. This reflects the fact that, at this juncture, gene therapy is still a radical, experimental therapy that may be justified only in this context.

Certain minimum criteria of potential efficacy also must be met. As a first step, it must be possible to deliver the therapeutic gene to the target cells of interest. After delivery, the introduced gene must be expressed at an appropriate level for the desired effect and for sufficient time for this effect to be achieved. Additionally, the delivery and expression of the therapeutic gene must be safe for the target cell and, by extension, for the individual being treated. From a conceptual standpoint, it must be recognized that these goals are all interrelated and, furthermore, that all of them must be addressed to rationally implement any gene therapy strategy. Clinical experience with gene therapy during the past decade shows that serious toxicity is not a problem. Most importantly, it is now evident that a variety of gene transfer maneuvers can alter favorably the cellular phenotype *in vitro*, although a fundamental limitation exists owing to an insufficient gene delivery and expression into target cells *in vivo*.

In practice, gene therapy implementation in human clinical trials has used two distinct strategies to meet the aforementioned criteria. In selected instances, target cells may be removed from the body, genetically modified extracorporeally, and then reintroduced into the patient. This *ex vivo* strategy has been applied in those contexts in which the technical capacity exists to readily harvest and manipulate the relevant target cell. As an alternative strategy, the *in vivo* approach involves directly delivering the therapeutic gene to the relevant target cells *in situ* in an intact individual. Whereas both approaches have been used in human clinical trials, the preponderance of strategies to date have employed the *ex vivo* approach. Although this method may offer certain advantages in selected contexts, it must be recognized that using this route presents the technical difficulty associated with accomplishing direct *in vivo* delivery.

The advantages of the *ex vivo* approach are that it allows gene transfer to the target cells in a defined, *in vitro* setting, in which delivery efficiencies may be optimized. This approach also allows the modified cells to be characterized from the standpoint of safety before they are reintroduced to the patient. Despite these advantages, this method may be limited to very select settings in which target cells can be propagated *ex vivo*; at present, this is viable for a very limited set of tissue types. The *in vivo* approach in theory overcomes this limitation of target tissue accessibility. Delivery *in vivo*, however, is fraught with considerably greater complexities than the *ex vivo* approach. Thus, the gene transfer vector in the direct-delivery approach must achieve delivery in the context of significant host barriers, including humoral, reticuloendothelial, and immunologic factors. This dichotomy highlights the reason why the

Table 33-1 ■ CLINICAL USE OF GENE TRANSFER SYSTEMS

TYPE	VECTOR SYSTEM	CLINICAL TRIALS*	DISTINGUISHING FEATURES
Nonviral	Liposomes	30	Repetitive administration feasible, inefficient gene delivery, transient expression
	Naked DNA or RNA (injection, gene gun, electroporation)	6	Easy preparation, inefficient gene delivery, transient expression
	Molecular conjugates	—	Flexible design, inefficient gene delivery, transient expression, unstable in vivo
Viral	Retrovirus	63	Integrates into the chromosome of dividing cells, unstable in vivo
	Adenovirus	34	Highly efficient in vivo; production in large numbers makes infection of tissues more efficient; tropism can be modified; induces potent inflammation and immunity
	Poxvirus (vaccinia)	15	Extensive clinical experience with parent virus; large insert capacity; induces potent inflammation and immunity
	Adeno-associated virus	1	Nonpathogenic; integrates into the chromosome; low insert capacity; difficult to scale-up
	Herpes simplex virus	1	Highly efficient in vivo; very large insert capacity; cytotoxic
	Chimeric vectors (e.g., Ad/Retro)	—	Combine features of component genetic vectors
	Lentivirus	—	Integrates into the chromosome of <i>both</i> dividing and nondividing cells; not yet available as a well-characterized production system

\*Registered in the NIH Office of Recombinant DNA Activities at the end of 1998.

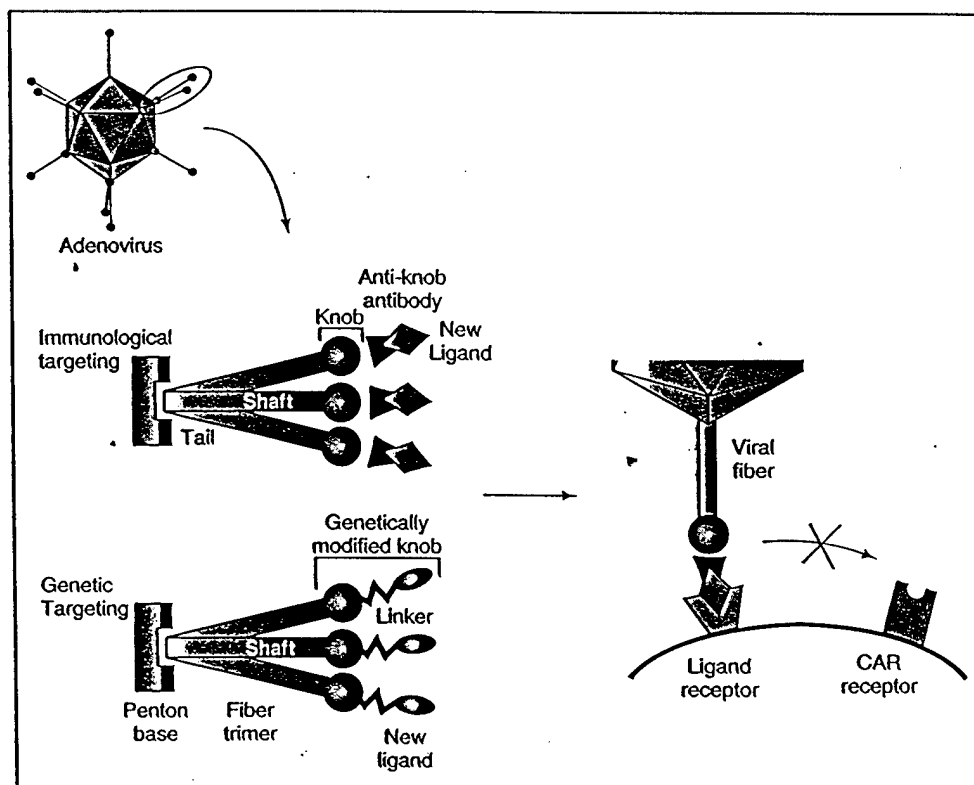
ability to accomplish in vivo gene delivery currently represents the greatest challenge to implementing gene therapy strategies. Earlier protocols were principally of the ex vivo type and relied on recombinant retroviruses as gene transfer vehicles.

The technology to derive recombinant retroviruses that can efficiently transfer genes has been sufficiently developed that these vectors have been used for a majority of human protocols (Table 33-1). They can accomplish effective gene transfer to a variety of target cells despite being rendered replication incompetent by genomic deletions. In addition, because these viruses are integrative, they can produce permanent genetic modifications of target cells with the consequence of long-term heterologous gene expression. Whereas the vectors are suited for ex vivo modification of target cells, a variety of limitations have restricted their use in strategies to accomplish direct, in vivo gene transfer. The retrovirus requires proliferative target cells to mediate effective gene transfer. One exception are lentiviruses, the class of retrovirus that includes hu-

man immunodeficiency virus, which can integrate also in non-dividing cells. An additional obstacle for retroviruses is the high susceptibility of the virus particle to humoral factors that ablate its gene-transfer capacity. Thus, the basic biology of recombinant retroviruses has been an additional factor restricting initially implemented gene therapy protocols to strategies using ex vivo methodologies.

To circumvent the limitations associated with recombinant retroviruses, alternative vector systems have been developed (see Table 33-1). These systems include both non-viral and viral approaches to accomplish gene transfer. In both of these approaches, the goal is to develop a system that can deliver genes in vivo after systemic administration. This development is a step toward deriving a "targetable-injectable" vector—a vector that can deliver therapeutic genes selectively to target cells after direct, in vivo administration. The development of such a vector system would have two very important consequences for potential gene therapy strategies: (1) it

FIGURE 33-1 ■ Methods to modify the adenoviral cellular tropism. Viral tropism is determined by the fiber and its recognition region in the terminus, or "knob" (dotted circle). Binding of the virus to target cells can be modified via either immunologic or genetic methods. Immunologic targeting involves the attachment of molecular conjugates incorporating antibodies against the fiber knob and ligands specific for cognate receptors in target cells. Genetic targeting involves the genetic modification of the fiber gene to generate fiber chimeras with novel ligand specificity. Note that in both cases, binding capacity to a non-native target cellular receptor is accompanied by ablation of the endogenous binding to the adenovirus primary receptor (coxsackievirus and adenovirus receptor [CAR]).



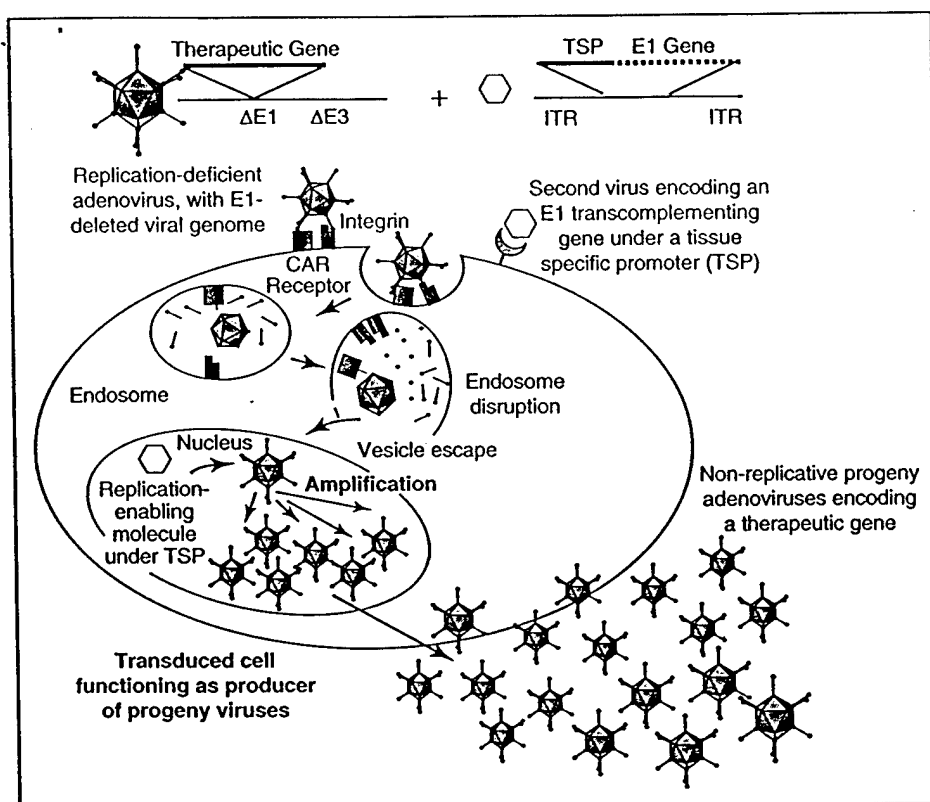


FIGURE 33-2 ■ Amplification of gene transfer by replicative viral vector systems. Replication-deficient adenoviruses can be genetically complemented *in vivo* in cells co-infect with a second vector that encodes the deleted replication-enabling molecules. Tissue- or tumor-specific promoters can regulate the expression of these proteins, thus limiting the occurrence of viral replication to the target tissue. Progeny virus leads to infection of neighboring cells, increased local viral inoculum, and augmentation of therapeutic gene expression.

would allow the implementation of a variety of gene therapy strategies targeted to cell or tissue types that cannot currently be modified *ex vivo*, and (2) it would allow gene therapy to be carried out in a context other than its present restriction to highly specialized tertiary care medical centers possessing the support facilities involved in *ex vivo* approaches.

A variety of nonviral systems have been developed and used in strategies to directly deliver genes *in vivo*. Liposomes are artificial lipid bilayer vesicles containing the foreign deoxyribonucleic acid (DNA). Their design overcomes the potential safety hazards associated with viral gene sequences contained in the viral vectors. Whereas initial formulations were associated with significant target cell toxicity, newer agents appear more promising in this regard. These vectors, delivered to select target organs after direct *in vivo* delivery, have been used in human clinical trials targeting pulmonary disorders and a wide variety of malignancies. Despite this systemic stability, delivery is at present non-specific, because the liposomes lack any mechanism to achieve targeting. This goal is the principal logic behind the design of molecular conjugate vectors. These synthetic molecules exploit the endogenous cellular receptor-mediated pathway to transfer genes. Their design characteristics allow a degree of targeting not available in other systems,

although a limited systemic stability may undermine the realization of that ability.

Non-retroviral viral vectors being actively developed include recombinant adenoviruses, the parvovirus adeno-associated virus (AAV), and vectors based on the herpes simplex virus. Adenoviruses have been evaluated for their ability to be directly delivered *in vivo* to a variety of organ systems, including the lung, liver, brain, and vasculature. Extensive characterization of the virus has made possible the introduction of numerous modifications in its genome and biochemical composition that overcome some of its limitations. Principal among them are the genetic and antibody-based modifications of the viral tropism (Fig. 33-1), the design of replicative vectors (Fig. 33-2), and the incorporation of immunomodulatory genes. The AAV vector is less highly developed but has also been associated with practical problems, including a very limited heterologous DNA-carrying capacity as well as very low viral titers. Herpes simplex vectors were developed for highly efficient gene transfer into neuronal tissues, but replication-deficient vectors with high capacity and low cytotoxicity have recently found utility in other contexts, such as bone marrow-derived cells and malignant tissues.

The rapid appearance of alternative systems, including target-

Table 33-2 ■ GENE THERAPY APPROACHES FOR THE TREATMENT OF CANCER

STRATEGY	CLINICAL TRIALS*	MOLECULAR MECHANISM OF ANTICANCER EFFECT
Mutation compensation	6	Inhibition of expression of dominant oncogene
	18	Augmentation of deficient tumor-suppressor genes
	2	Abrogation of autocrine growth factor loops (single chain antibodies)
Molecular chemotherapy	36	Selective delivery of toxin or toxin gene to cancer cells
Genetic immunopotential	54	<i>In vitro</i> transduction—augmentation of tropism or cell killing capacity of tumor-infiltrating lymphocytes; genetic modification of irradiated tumor cells
	47	<i>In vivo</i> transduction—administration of costimulatory molecules or cytokines; immunization with virus encoding tumor-associated antigens
Viral-mediated oncolysis	2	Tumor cell lysis by viral vector replication.

\*Registered in the NIH Office of Recombinant DNA Activities at the end of 1998.



vectors built on available viral-based systems (see Fig. 33-1), predicts that additional strategies can achieve the central goal of targeted gene delivery after the targeted vector is administered directly in vivo. Furthermore, chimeras have been designed that adopt favorable features of parental vectors.

Initial strategies to accomplish gene therapy were designed for inherited genetic disorders. Since the first human clinical trial of gene therapy in 1990, a variety of genetic diseases—ADA deficiency, cystic fibrosis, Gaucher's disease, Canavan's disease, hyperlipidemia—have been treated using gene therapy. Specific strategies to treat a variety of other disorders are being developed, but the central problem of delivering genes to target cells has limited more general use of these methods for inherited genetic disorders.

Gene therapy is also rational in the context of acquired genetic disorders and thus has been used to target cancer; the overwhelming number of human gene therapy protocols in trial have been for cancer therapy. A variety of approaches have been developed based on the molecular pathogenesis of cancer. Specific strategies include the following: (1) a wild-type tumor suppressor gene for recessive oncogene mutations, (2) inhibitory gene constructs for dysregulated or overexpressed dominant oncogenes, (3) toxin genes selectively delivered to cancer cells to eradicate them, and (4) immunomodulatory genes to increase the immunogenicity of tumors (Table 33-2). Each of these specific strategies has a counterpart in human protocols that have been approved for clinical trials. Interestingly, these strategies have begun to be applied to inhibit or destroy the tumor vasculature.

Gene transfer may represent a therapeutic strategy applicable to contexts outside of genetic diseases. The ability to modulate specific patterns of gene expression may represent a viable option in certain inflammatory, vascular, and immune-mediated disorders. In this regard, specific genetic methods of abrogating selected patterns of gene expression have been shown to alter disease pathogenesis in models of inflammatory and fibrotic lung disease, rheumatoid arthritis, postischemic hepatic injury, postcatheterization arterial stenosis, peripheral artery disease, and organ graft rejection. Gene therapy thus offers a means for highly selected ablation of specific gene expression patterns. Using techniques of "anti-sense" inhibition or "single-chain antibodies," the informational flow from genes through the level of protein is interrupted based on sequence-specific hybridization or by antibody-antigen binding specificity. This strategy offers the potential to intervene with a level of specificity at multiple sites in the gene expression pathway not achievable using classic pharmacological therapies.

Thus, gene therapy may ultimately represent another form of pharmacotherapy, albeit a form with mostly improved potentials based on exploitation of the exquisite specificity offered by the cellular apparatus of gene expression. As vector technology achieves the basic ability to deliver therapeutic genes quantitatively, and specifically, into target cells, it is anticipated that promising results already observed in preclinical studies will translate quickly into the clinic for amelioration of a variety of diseases.

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offspring. Errors in chromosome behavior and structure are the cause of a wide range of clinical syndromes.

Every nucleated somatic cell in the human has a complete genome of about  $6 \times 10^9$  base pairs of DNA, with an uncoiled total length of approximately 2 meters. The genome is packaged by supercoiling into 46 chromosomes, consisting of 22 pairs of homologous chromosomes (identical in regard to morphology and constituent gene loci) and 1 pair of sex chromosomes (X and Y), one partner of each pair being derived from the mother and one from the father. The 46 chromosomes in metaphase vary in length from 2 to 12  $\mu\text{m}$ . The genes are arranged along the chromosomes in linear order, with each gene having a precise position or locus. Genes that have their loci on the same chromosome are said to be *syntenic*; genes that are close together on the same chromosome and tend to travel together during meiosis (little crossing-over) are said to be *linked*. Alternate forms of a gene that occupy the same locus are called *alleles*. Any one chromosome bears only a single allele at a given locus, although in the population as a whole there may be multiple alleles, any one of which can occupy that specific locus.

**CELL DIVISION.** The number of chromosomes found in somatic cells is constant and is termed the *diploid* ( $2n$ ) number. Each gamete, however, has only half the diploid number and is said to be *haploid* ( $n$ ). To maintain this regularity, two types of cell division occur: *mitosis*, which is the cell division occurring in somatic tissues during growth and repair, and *meiosis*, which is the specialized form of cell division occurring when gametes form.

**MITOSIS.** The function of mitosis is to distribute and maintain the continuity of the genetic material in every cell of the body. This process consists of a number of different phases, which results in an equal distribution of the chromosomes to the two daughter cells. The cell cycle has four stages: mitosis (M), gap<sub>1</sub> (G<sub>1</sub>), synthesis (S), and gap<sub>2</sub> (G<sub>2</sub>). The G<sub>1</sub> phase follows mitosis, during which RNA and protein synthesis occurs. S is the period during which DNA replication takes place and the DNA content of the cell doubles, and G<sub>2</sub> is the period during which energy requirements for cell division are built up and any repair of errors in DNA synthesis takes place. Mitosis has four phases: (1) prophase, during which the duplicated chromosomes condense into microscopically visible bodies; (2) metaphase, during which the chromosomes continue to contract and line up on the metaphase plate; (3) anaphase, when the chromatids of the chromosomes separate at the centromeres; and (4) telophase, when the separated chromatids gather and the cell divides.

**MEIOSIS.** This process occurs only during the formation of the gametes and results in four daughter cells, each with the haploid number of chromosomes. In males, each primary spermatocyte forms four functional spermatids that develop into sperm, whereas in females, each oocyte forms only one ovum, the remaining products of meiosis being nonfunctional polar bodies.

Processes fundamental to meiosis include chromosome pairing, chromosome crossing-over, and chromosome segregation. These processes result in halving the chromosome number, regular distribution of chromosomes to daughter cells, and independent assortment of the genetic material from both the cross-over events and maternal-paternal homologue distribution in meiosis I. The ultimate result ensures genetic variability.

**METHODS FOR THE PREPARATION OF CHROMOSOMES.** Because non-dividing chromosomes cannot be analyzed, live dividing cells are required for chromosome analysis. The cell type most commonly used is the mitogenically stimulated peripheral blood lymphocyte. Skin fibroblasts, bone marrow cells, amniotic fluid cells, chorionic villus cells, and tumor cells are also used for special tests. Dividing cells are accumulated at metaphase. To accomplish this, a drug (Colcemide) that destroys the mitotic spindle is added to the culture medium toward the end of the culture period. The cells are subjected to hypotonic treatment followed by fixation and spreading on microscope slides. The slides are then stained.

Staining techniques may result in either a non-banded or a banded appearance of the chromosomes. Laboratories today use at least one of several banding techniques; this results in a great deal of additional information. These methods provide a means to precisely identify each chromosome and extra or missing chromo-

## 34 CHROMOSOMES AND THEIR DISORDERS

R. Ellen Magenis

Cytogenetics is the study of chromosomes and their behavior as they relate to transmission of the genetic material from parent to

# **Anti-neoplastic Effect of Anti-erbB-2 Intrabody is not Correlated with scFv Affinity For Its Target**

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**Running Title:** Anti-neoplastic Effect of Anti-erbB-2 Intrabodies

**Keywords:** Gene Therapy, single-chain antibody, erbB-2, cytotoxicity

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## **Abstract**

Intracellular single-chain antibodies (scFv) have emerged as a powerful method to knock out expression of oncoproteins. We have previously demonstrated that scFvs directed against a variety of molecular targets induce specific toxicity in tumor cells. Recently, the utility of an anti-erbB-2 scFv has predicated its evaluation in a phase I gene therapy clinical trial. The utility of scFv as an intrabody is closely linked to its interaction with a target, limiting the contribution of the latter to the neoplastic phenotype. In this study we sought to determine if improvement in the affinity of the scFv for its cognate target could improve the efficiency of intrabody-mediated oncoprotein knockout. We compared in erbB-2 positive and negative tumor cells the function of plasmids encoding a newly developed C6.5 anti-erbB-2 scFv, which has a 1000-fold higher affinity, with our original e23 anti-erbB-2 scFv. Intracellular sFv expression, target binding, and tumor cell cytotoxicity were found to be similar in all conditions tested, including dose-response studies with limiting dilutions of the scFv. On this basis, we have concluded that the anti-neoplastic effect of anti-erbB-2 intrabody is not correlated with scFv affinity for its target.

## Introduction

The cancer gene therapy of mutation compensation is based upon rectification of the genetic lesions etiologic of neoplastic progression. For the context of dominant oncogenes, methods have been developed to achieve abrogation of the dysregulated cellular pathways.<sup>1-11</sup> Such interventions have been endeavored at the transcriptional level of gene expression employing triplex, antisense or ribozyme approaches.<sup>4-8;10-13</sup> In addition, functional knockout at the level of protein expression has been achieved via dominant negative mutation, as well as intrabody methods.<sup>14-26</sup> This later approach is based upon intracellular expression of single chain antibodies (scFv) to sequester oncoproteins during their biosynthesis, thus preventing their further maturation.<sup>14;17-21;23;24;27-33</sup>

On this basis, intrabody knockout of oncoproteins has found broad utility for a variety of molecular targets. In this regard, intrabody knockout of diverse classes of oncoproteins has been reported including overexpressed growth factor receptors (erbB-2, EGF-R), cell cycle proteins (cyclin D1, p53), viral oncoproteins (ras, HBV, LMP1, HPV E6/E7) and anti-apoptosis proteins (Bcl-2).<sup>2;17;19;23;29-31;34-41</sup> In this context, diverse antineoplastic effects mediated by intrabodies have been reported including tumor cell specific cytotoxicity, chemosensitization and radiosensitization.<sup>40;42;43</sup> These utilities have predicated the evaluation of intrabodies in the context of a human clinical gene therapy trial whereby an anti-erbB-2 intrabody has been studied in a phase I protocol for erbB-2 overexpressing carcinoma of the ovary.<sup>44</sup>

The functional basis of intrabody utility is closely linked to the ability of the scFv to interact with its target and prevent its maturation, thus limiting its contribution to the neoplastic phenotype. Thus, for optimal function an intrabody must be expressed in target cells at high level, achieve appropriate subcellular localization permitting interaction with its target, and maintain high affinity sufficient for effective capture of the corresponding oncoprotein. To this end, we have explored the utility of efficient gene delivery vehicles in the context of intrabody knockout strategies.<sup>29,43,45-47</sup> As well, our group, and others, have confirmed the key importance of employing heterologous cellular processing signals for achieving effective intrabody colocalization with target oncoproteins.<sup>2,19;23;28-31,36;37;39;40;46;48</sup>

In the present study we sought to determine if improvement in the affinity of the parent scFv could offer an additional means to improve the efficiency of intrabody-mediated oncoprotein knockout.

We have previously reported on the utilities of an anti-erbB-2 intrabody approach in the context of erbB-2 overexpressing tumor target.<sup>23,29-31,38;46,48-50</sup> In these studies, our intrabody was derived from the anti-erbB-2 scFv e23. In this regard, screening of a phage display library and site-directed mutagenesis has been employed to obtain an anti-erbB-2 scFv such that this new derivative anti-erbB-2 scFv, C6.5, possesses an affinity for its target 1000 times greater than e23.<sup>51-53</sup> We thus employed this scFv to determine the degree to which affinity differential predicated utility for intrabody knockout approaches. Our results demonstrated that affinity enhancement of the parent scFv does not necessarily predict an improvement in an intrabody-mediated antineoplastic effects.

## **Material and Methods**

### ***Cell Lines***

The human ovarian cancer cell line SKOV3i.p1 was a generous gift from Janet Price (MD Anderson Cancer Center, Huston, TX); the prostate cell line DU145, human cervical cell line HeLa, human lung cancer cell line A549, and the breast cancer cell line MCF-7 were all obtained from ATCC (Rockville, MD) and maintained in DMEM/F12 (DU145, SKOV3.ip1), DMEM (HeLa) or RPMI (A549 & MCF-7) (Mediatech, Inc, Herndon, VA) supplemented with L-glutamine (300 ug/ml), penicillin (100 I.U./ml), streptomycin (25 ug/ml) and 10% fetal calf serum at 37°C in a humidified 5% carbon dioxide atmosphere.

### ***Plasmid construction and preparation***

The anti-erbB-2 scFv C6.5 and anti-erbB-2 scFv e23 were cloned into the plasmid pSTCF.KDEL, which contains an endoplasmic reticulum retaining signal, giving rise to the plasmids pSTCF.C6.5 and pSTCF.e23. The plasmid pSTCF.LMP1 was obtained from a similar plasmid encoding the control anti-LMP1 scFv. The construction was as follows. The scFv expression plasmid pSTCF.KDEL was derived from pSecTagC (Invitrogen, San Diego, CA). This vector, derived from the plasmid pcDNA3, encodes an IgK leader sequence that targets encoded proteins into the secretory pathway, followed by a polylinker, an in-frame myc tag epitope, and a KDEL sequence. Expression is driven by the citomegalovirus (CMV) promoter. In order to allow in-frame cloning of SfiI / NotI flanked scFvs from pCANTABS5E (Pharmacia Biotechnology) and other scFv phage display vectors, the pSecTagC vector was digested with SfiI and ApaI. The

following complementary oligonucleotides were then annealed and ligated into the digested plasmid vector: 5' CGG CCG TTA ACG CGG CCG CCG GGC C 3' and 5' CGG CGG CCG CGT TAA CGG CCG GCT 3'. The resulting construct was verified by sequencing, and named pSTCF. To construct pSTCF.KDEL, the pSTCF plasmid was digested with NotI and ApaI. The following complementary oligonucleotides were then annealed into the digested plasmid vector: 5' GGC CGC CGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GAA GAA AAG ACG AAC TCT GAG GGG TTC CGC C 3' and 5' CTC ACA GAG TTC GTC TTT TTC TGA ATT CAG ATC CTC TTC TGA GCT GAG TTT TTG TTC GGC 3'. These oligonucleotides replaced the His tag present in the original pSTCF construct with an internal myc tag and the ER retention sequence SEKDEL. Lastly, we cloned our e23 scFv and C6.5 scFv in this construct using SfiI and NotI restriction sites. The plasmid for the control gene pSTCF.LMP1 was provided by Dr. Alain Piche,.

### ***Plasmid transfection***

All transfections were performed using the adenovirus/polylysine system (Ad/pL), as previously described.<sup>54</sup> This vector, consisting of a replication-incompetent adenovirus conjugated to poly-L-lysine, has been previously shown to achieve highly efficient transfection of SKOV3.ip1, DU145, A549 and HeLa cells. Briefly, 6 ug of plasmid DNA was conjugated with an optimized amount of AdpL vector and 4 ug of free poly-L-lysine, constituting one complex. Complexes were diluted in culture medium containing 2% FCS and added to cells in 6 well plates at a ratio of 1 complex per 1 million cells. After 1 hour, an equal volume of complete medium was added to the wells. Cells were then harvested and analyzed at various time points post-transfection.

For the study of cytotoxicity, a 2.5 ul fraction of a single AdpL complex was mixed with 100 ul of the appropriate 2% media and incubated with the cells for 1 hr. At that time, 100 ul of complete media was added. Cells were then incubated for 48-96 hr after transfection, an assayed as described below.

### ***Immunoblotting***

To assess the expression of protein by Western blot, cells were lysed for 30 minutes on ice with RIPA buffer (150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA and 50 mM Tris, pH7.4), by adding 300 ul per well in a six-well plate. Cells were then scraped, collected in microfuge tubes, and spun at 14,000 rpm for 15 minutes at 4°C. Supernatants were removed and protein concentration was assessed using the BioRad protein assay (BioRad, Hercules, CA) according to the instruction provided by the manufacturer. After mixture with 2X Gel Loading Buffer, 50 ug of total protein were separated on 12% SDS-PAGE gel (scFv) followed by transfer to PVDF membrane (BioRad). Membranes were probed with the anti-myc tag antibody 9E10 (Invitrogen, Carlsbad, CA) at a dilution of 1:2500, for scFv detection. HRP labeled goat-anti-mouse was used at a dilution of 1:5000 as secondary antibodies. Western blots were developed using the Renaissance reagent system (Dupont, Boston, MA).

### ***Immunoprecipitation***

To evaluate the interaction of the erbB-2 protein with the anti-erbB-2 scFv the whole cell lysate from DU145 cells transfected with the erbB-2 scFv plasmid was prepared as described above. It was then immunoprecipitated with a monoclonal human erbB-2 antiserum (Ab-3, Calbiochem). To this end, the antibody was mixed with 500 ul of



immunoprecipitation (IP) wash buffer (50 mM Hepes, pH7.4, 150 mM NaCl, 1 mM sodium vanadate, 1% Triton X-100, 10 % glycerol), 500 ul of Gold Buffer (2 mM Tris, 150 mM NaCl, gelatin, 0.1 % BSA, 0.5% Tween-20, 0.02% sodium azide), and 30 ul of protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), and rotated overnight at 4°C. The beads were then washed by brief centrifugation, followed by resuspension in 1 ml of IP wash buffer. This step was repeated 5 times. The beads were then resuspend with 500 ul of the cell lysate and rotated at 4°C for 2 hours. After washing as above, beads were resuspended in 100 ul of 1X SDS-PAGE sample buffer and placed on a 100°C heat block for 10 minutes. The samples were then evaluated by SDS-PAGE on a 12% gel, immunoblotted on to PVDF membranes (BioRad, Hercules, CA), and probed with anti-myc tag sera, as described above.

### ***Cellular cytotoxicity***

The effect of expression of the anti-erbB-2 scFv on cell growth and viability was determined employing the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation/Cytotoxic assay (Promega, Madison, WI). This MTS-based colorimetric assay measures the ability of viable cells to convert a tetrazolium salt to the formazan compound. To accomplish this analysis, cells were seeded into 96-well cell culture plates at a density of 5,000 cells/well. Transfer of the scFvs-encoding plasmids pSTCF.e23, pSTCF.C6.5 and pSTCF.LMP1 was performed employing the Ad/pL system, as described above. A 2.5 ul fraction of a single Ad/pL complex was mixed with 100 ul of appropriate 2% media and incubated with the cells for 1 hr, at which point 100 ul of complete media was added. The plate was subsequently incubated for up to 96 hr. The Cell Titer 96 kit was then used according to the manufacturer's instructions. Percent

viability was calculated by the following formula:  $[(\text{Infected cells} - \text{blank}) / (\text{Uninfected cells} - \text{Blank})] \times 100$ .

## Results

Plasmids were derived for intracellular eucaryotic expression of the anti-erbB-2 scFvs e23 and C6.5. Plasmid elements include an IgK leader sequence, a minor terminal leader sequence, a carboxy-terminal myc epitope tag, and a KEDEL signal for the achievement of retention of the expressed protein in the endoplasmic reticulum (Fig. 1). Constructions were confirmed by digestion with restriction endonucleases and restriction analyses (data not shown). High efficiency transfection of this plasmid construct was achieved via adenovirus-polylysine conjugate (Ad/pL) employing described techniques.<sup>54</sup> Initial analyses sought to confirm that the e23 or C6.5 intrabody construct could achieve comparable levels of intracellular expression. Such an outcome would thus allow attribution of any differential biological effects to the affinity difference of the scFvs. The initial study employed the erbB-2 negative human cervical cell line HeLa. Transfection of the HeLa with Ad/pL conjugate containing the e23 plasmid pSTCF.e23 yielded a 35-kDa band when cell lysates were immunoblot analyzed with an anti-myc antibody (Fig. 2A). The appropriate size of the anti-erbB-2 intrabody was confirmed by comparison to the control plasmid pSTCF.LMP1. This construct encodes a scFv to a target irrelevant to the current analysis, the LMP1 protein of EBV. Of note, the plasmid pSTCF.C6.5 resulted in expression of an appropriate size protein in amounts equal to that achieved by transfection of pSTCF.e23. Thus, the plasmid-driven expression of the scFv e23 and C6.5 are associated with comparable levels of anti-erbB-2 intrabody.

We next sought to validate that the intracellular expression of the anti-erbB-2 scFv was capable of achieving interaction with that target. For this analysis, target erbB-2 negative HeLa, and positive DU-145, cell lines were transfected with the plasmid constructs as before. Cell lysate from transfected cells were then immunoprecipitated with anti-erbB-2 antibody and subjected to denaturing gel resolution followed by immunoblot with anti-myc antibody. In this study, both of the anti-erbB-2 scFvs were detected from transfection of the erbB-2 positive cell line DU-145 (Fig. 2B). This finding confirmed that both anti-erbB-2 antibodies possessed the capacity to recognize their cognate target in the context of intracellular expression. As control, similar analysis of the erbB-2 negative HeLa cell line did not exhibit any retained intrabody, confirming that anti-erbB-2 specificity of the intracellularly expressed scFv. To this point, we had thus established that the anti-erbB-2 scFv were both capable of recognizing their cognate target in the intracellular context and were both associated with comparable level of intracellular expression of intrabodies.

On this basis, it was thus relevant to directly compare the efficacy of the intrabodies in achieving antineoplastic effects. In this regard, our previous studies have demonstrated that intracellular expression of anti-erbB-2 scFv can induce a direct cytotoxicity in erbB-2 expressing tumor cells.<sup>23,29-31,38,46,48-50</sup> We thus sought to directly compare the e23 and C6.5 intrabodies for this capacity in the context of the erbB-2 positive prostate carcinoma cell line DU-145. Cells were transfected with the conjugate Ad/pL containing either pSTCF.e23 or pSTCF.C6.5 and analysis carried out at different times post-transfection for viable cells number. In this study it could be seen that the e23 intrabody elicited significant cytotoxicity compared to Ad/pL conjugates containing the control

plasmid pSTCF.LMP1 (Fig. 3). Of note, the C6.5 intrabody induced a level of cytotoxicity comparable to the e23 antibody. The level of plasmid delivered in this study was not sufficient to induce quantitative tumor cell cytotoxicity. On this basis, any differential cytotoxicity of the test intrabodies would have been apparent in this study. Thus, despite the differential target affinity of the e23 and C6.5 scFvs, their derivative intrabodies were of comparable efficiency in inducing tumor cell cytotoxicity.

Two additional studies were endeavored for defining any differential of the intrabodies. First, we have previously found that the level of erbB-2 in target cells is an important predictor of anti-erbB-2 intrabody-mediated cytotoxicity.<sup>29</sup> We thus carried out paired transduction of the e23 and C6.5 encoding plasmids in the context of target tumor cell lines expressing variable levels of erbB-2. As before, Ad/pL transfection of the plasmid was followed by analysis of the cell survival. In this study, there was no significant difference in intrabody-mediated cytotoxicity for the e23 and C6.5 intrabodies (Fig. 4). Thus, even in the context of variable level of the erbB-2 target, the differential affinity of the e23 and C6.5 scFvs did not predict differentiated antitumor efficacy of the derivative intrabody.

In addition, we explored the potency of the intrabodies in the context of a limiting dilution analysis. In this regard, our previous studies have demonstrated a threshold effect for intrabody-mediated cytotoxicity.<sup>50</sup> In these studies, it was shown that a requisite level of intrabody plus target was required to elicit tumor cell cytotoxicity. We thus hypothesized that the higher affinity of the C6.5 scFv for its target might be manifested if limiting levels of the intrabody were expressed. Target DU-145 cells were thus transfected with various levels of the plasmid pSTCF.e23 and pSTCF.C6.5. Analyses for cytotoxicity

were endeavored as before. Whereas a threshold effect could be noticed for cytotoxicity, this threshold levels did not differ for the test intrabodies (Fig. 5). It was thus apparent that differential efficacy of the intrabodies, manifested as dose-related potency, could not be attributable to the differential binding affinities of the parent scFvs.

## Discussion

Oncogene knockout represents an attractive cancer gene therapy approach whereby a highly targeted intervention may be achieved.<sup>14-17,26</sup> Based on this concept, a number of methods have been endeavored to achieve selective gene product abrogation as a means to revert the transformed phenotype. These interventions have included antisense and ribozymes designed to interrupt oncogene expression at the transcriptional level, as well as dominant negative molecules and intrabodies to achieve direct oncoprotein knockout.<sup>14-17,26</sup> The capacity of these agents to achieve a highly selective anti-tumor effect has allowed the realization of therapeutic index profiles warranting translation into the human clinical context.<sup>44</sup>

In this context, intrabodies offer a number of practical advantages, which have warranted their further study. Chief among these advantages is the facile means whereby anti-oncoprotein scFvs may be derived. Such methods have included genetic techniques of deriving cDNA directly from hybridomas as well as biopanning of phage scFv libraries and others.<sup>51;2,17,19,23,29,36,37,39-41,52,53,55</sup> These methods have allowed direct isolation of scFv against virtually any subcellular target protein. Thus, unlike antisense and ribozymes whereby specific recognition, and ablation, of target oncogenes sequences must be empirically determined, scFv may be reliably derived from accessible substrate systems for their employ as intrabodies.

The knockout utility of derived scFv in the intrabody context is thus subservient to expression of threshold levels at the appropriate subcellular locale and retained recognition of the cognate oncoprotein. In this regard, scFv may be directed to selected

subcellular compartments via heterologous processing signals, and this capacity has allowed knockout of oncoproteins within the nucleus, cytosol, mitochondrial membrane, and the endoplasmic reticulum.<sup>50</sup> Intracellular expression of scFv, however, is a parameter that cannot be predicted *a priori*. On this basis, limited panels of derived scFv must be directly screened in the context of eucaryotic cells for determination of the optimal expression profile. Subsequent to the achievement of threshold expression at the appropriate locale, the intrabody is presumed to interact with its target based on affinity dynamics derived from the parental scFv.

In the current study we sought to determine if affinity enhancement in the parental scFv would augment the anti-neoplastic efficacy of the derivative intrabody. In this regard, various techniques have been employed to improved scFv affinity for cognate targets.<sup>51-</sup>

<sup>53</sup> Such efforts have largely been endeavored in the context of the comparison of recombinant immunotoxins, whereby the affinity of the antibody clearly correlated with tumor targeting, and with the overall therapeutic efficacy. In our studies, increasing the binding affinity of the scFv did not translate into an improvement in intrabody-mediated tumor cell cytotoxicity induction. A variety of considerations may be invoked to explain this result. First, it is not feasible to actually determine the affinity of the intrabody for its target in the intracellular context; it is plausible that the affinity differential of the e23 and C6.5 scFv was not maintained at its ER site of intrabody residence. Alternatively, the co-localization of the intrabody and target erbB-2 within the confinement of the ER may allow interactions of a magnitude whereby affinity augmentation is irrelevant. In either event, these studies serve to highlight the degree to which a direct extrapolation of the utilities of scFv to the intrabody context does not have universal validity.



In our human trial we have employed an anti-erbB-2 scFv for erbB-2 overexpressing carcinoma of the ovary.<sup>44</sup> These studies, as well as preclinical studies in murine model systems, have served to highlight gene delivery into tumor cells as the major practical limitation in realizing the full benefit of intrabody approaches.<sup>56-57</sup> On this basis, intrabodies may be considered in the context of various other mutation compensation approaches, whereby direct tumor cell transduction is a requisite for anti-tumor action. As such, these various approaches require quantitative, or near-quantitative tumor cell transduction, to allow a therapeutic result. The recognition that no vector system can presently achieve this end thus belies the notion that intrabodies, or any mutation compensation approach, is currently rational for clinical cancer gene therapy applications. None-the-less, strategies to potentially improve biologic action, in the context of limited gene delivery, such as we have endeavored here, remain an area of legitimate pursuit to realize the conceptual benefits potentially offered by oncogene knockout approaches.

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## Figure Legends

**Figure 1. Coding region of plasmids pSTCF.e23 and pSTCF.C6.5.** Open reading frame derived the scFv e23 and C6.5 were constructed into the parent plasmid pSecTag3. The coding region of the parent plasmid contains an ATG start site, an IgK leader sequence, the scFv cDNA, and a myc epitope tag and KDEL signal for retention in the endoplasmic reticulum (ER), followed by a TAA stop signal.

**Figure 2. Intracellular expression of anti-erbB-2 scFv. A.** Comparison of expression levels of e23 and C6.5 intrabody in erbB-2 negative cell line, HeLa. Cells were transfected with plasmids encoding LMP-1, e23, and C6.5 intrabodies, and cellular lysates analyzed for myc epitope. Mock represents untransfected control cells. Arrow indicates migration at 35 kDa. **B.** Evaluation of the target recognition of the e23 and C6.5 intrabodies. ErbB-2 positive DU-145 and erbB-2 negative HeLa were transfected with the intrabody. Cell lysates were immunoprecipitated by the anti-erbB-2 antibody, resolved by denaturing gel electrophoresis and then probed for myc by immunoblot. Arrow indicates migration of 35 kDa.

**Figure 3. intrabody-induced cytotoxicity in DU-145 tumor cells.** Transfection of DU-145 was carried out with plasmid encoding anti-erbB-2 intrabodies e23 or C6.5. Controls included untransfected cells (mock), or plasmid encoding irrelevant intrabody LMP-1. The DU-145 cells were analyzed for viability at 36, 48, and 72 hours post-

transfection. Results are indicated as percentage of viable cells compared to untransfected controls. Experiments were performed in triplicates and results are indicated as mean  $\pm$  SEM.

**Figure 4. Anti-erbB-2 intrabody-mediated cytotoxicity in various erbB-2 positive cell lines.** The indicated erbB-2 positive human carcinoma cell lines were transfected exactly as indicated in Figure 3. Analysis was performed at 48 hours for cell viability. Experiments were performed in triplicate and results are indicated as mean  $\pm$  SEM.

**Figure 5. Dose-response analysis of anti-erbB-2 intrabody-mediated cytotoxicity for DU-145 cells.** The erbB-2 positive human carcinoma cell line DU-145 was transfected with variable doses of the plasmids encoding e23 or C6.5 intrabodies. Analyses for cell viability were accomplished as before. Experiments were performed in triplicate and results are indicated as mean  $\pm$  SEM.

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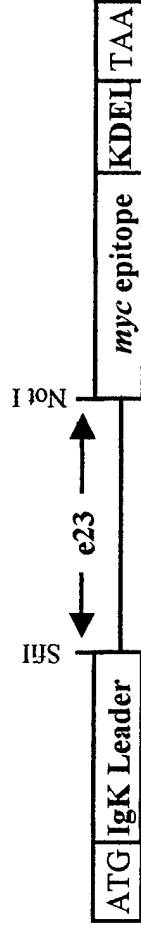


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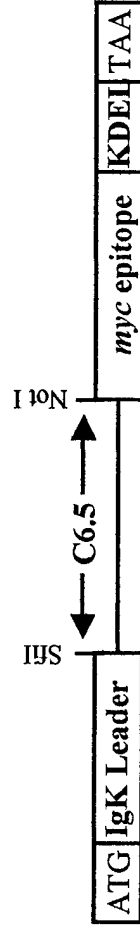
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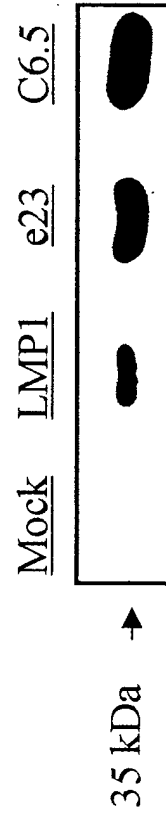
pSTCF. e23 sFv



pSTCF. C6.5 sFv



A.



**B.**

